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Covalent and non-covalent interaction of chymotrypsin with α_2-macroglobulin

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The pattern of covalent crosslinking between human α_2-macroglobulin (α_2M) and chymotrypsin has been investigated by chromatography and polyacrylamide gel electrophoresis in denaturing medium. Reaction with a single mol of chymotrypsin per mol α_2M results in the formation of a 95% covalent 1:1 chymotrypsin-α_2M complex and in the proteolytic cleavage of both 180 kDa monomers in one α_2M subunit. Proteolytic cleavage in the other α_2M subunit requires the presence of a second mol of chymotrypsin; part (20%) of the protease in the 2:1 chymotrypsin-α_2M complex thus formed appears to be non-covalently bound to the α_2M chains. Covalent binding is abolished when the reaction of α_2M with the protease is carried out in the presence of hydroxylamine. A single mol of the protease is then able to cleave all four 180 kDa monomers in α_2M.

Chymotrypsin; α_2-Macroglobulin; Covalent interaction; Non-covalent interaction; Electrophoresis

1. INTRODUCTION

Human α_2M is a tetramer of four identical subunits formed by the non-covalent association of two disulfide-bonded pairs [1–3]. Each subunit has two functional regions. The first is the site of limited proteolysis by bound proteases (bait region) and is located near the centre of the subunit peptide chains [1], the second region is a reactive group, namely a thiol ester [4,5], located 200 amino acid residues from the first. The inhibition reaction has been proposed to occur via a 'trap' mechanism [6] initiated by a limited proteolysis of the α_2M bait region followed by a conformational change during which the disruption of the thiol ester bonds occurs, leading to the appearance of activated glutamic acid residues which can covalently react with the entrapped protease [7]. In the present study, we show that the covalent binding of chymotrypsin to α_2M in a 1:1 molar ratio induces the transformation of only one of the two functional domains [8] of the inhibitor, whereas a non-covalent binding mode of the protease is able to transform the whole α_2M molecule, as estimated by the extent of proteolytic cleavage.

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Abbreviations: α_2M, α_2-macroglobulin; FITC, fluorescein isothiocyanate; I-AEDns, *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonate

2. MATERIALS AND METHODS

2.1. *Materials*

Human α_2M was prepared by Zn^{2+} affinity chromatography [9] and filtered on an ACA 22 column as a final step of purification. Bovine pancreatic chymotrypsin was purified by chromatography on a DEAE-trisacryl column equilibrated

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with 10 mM Tris-HCl, pH 8.0, and labelled by fluorescein isothiocyanate as in [10].

2.2. *Methods*

All enzymatic and spectrophotometric measurements were made in 20 mM Hepes, 50 mM NaCl, pH 7.10.

Chymotrypsin activity was evaluated using the chromogenic substrate Suc-Ala-Ala-Pro-Phe-pNA (Serva).

Substitution of free cystein sulfhydryl groups in chymotrypsin-α_2M complexes by I-AEDns was achieved as in [10].

α_2M and chymotrypsin were radioiodinated using the Bolton Hunter reagent (NEN) as follows: proteins at 5 mg/ml in 80 mM Hepes, pH 7.8, were added to about 0.5 mCi of the reagent and left at room temperature for 10 min. The ^{125}I-labelled proteins were then isolated by Sephadex G-25 filtration.

The estimation of covalently bound chymotrypsin was performed using FITC-chymotrypsin. Protease-α_2M complexes were filtered on a Sephacryl S-300 column under denaturing conditions and the peak area corresponding to FITC-labelled protease comigrating with high-M_r fragments of α_2M estimated [11].

Polyacrylamide gel electrophoresis was performed in 5% acrylamide, pH 8.8, 0.05% SDS. Samples were prepared by adding a given amount of chymotrypsin to 60 μl of 3 μM α_2M. After 10 min of incubation, 5 μl of diisopropylfluorophosphate (0.5 M in dioxane) were added, followed 10 min later by 5 μl of 10% SDS, and 5 μl mercaptoethanol. The mixtures were heated to 45°C for 1 h and submitted to electrophoresis. After staining with Coomassie blue, the gels were stained and cut in 2 mm slices and the radioactivity of each slice measured in a gamma counter.

Fluorescence was measured using a Kontron SFM-25 fluorimeter equipped with a thermostated cell holder and polarization accessories. Energy transfer efficiencies within the α_2M-protease complexes [11,16] were determined as $E = 1 - \Phi_{DA}/\Phi_D$ from the fluorescence quantum yield of the complex containing the donor only (Φ_D = I-AEDns-labelled α_2M + unlabelled chymotrypsin) and of the complex containing both the donor and acceptor dyes (Φ_{DA} = I-AEDns-labelled α_2M + FITC-labelled chymotrypsin). The polarization values of the FITC-labelled conjugates were determined with excitation at 475 nm and emission at 520 nm.

Electron microscopy was performed as in [12].

3. RESULTS

3.1. *Influence of the covalent and non-covalent binding mode of chymotrypsin to α_2M on the extent of bait region cleavage*

Fig.1 shows the electrophoretic pattern of chymotrypsin-α_2M complexes formed at different protease-α_2M molar ratios. The two major bands observed correspond to the well known [13] conversion of the 180 kDa chains (C) of α_2M into 90 kDa fragments (F) by limited proteolysis. Fainter bands also appeared in two pairs (A + B) and (D + E) at apparent molecular masses of about 200 and 100 kDa, respectively. These bands are found to contain ^{125}I-chymotrypsin and can be then considered as covalent conjugates of protease fragments generated by the reduction of disulfide bridges [14] within the chymotrypsin molecule with the 180 and 90 kDa peptide chains of α_2M.

When ^{125}I-labelled α_2M is used, the bulk of the radioactivity is found to co-migrate with bands (C) and (F) whose relative amounts allow one to determine the extent of proteolytic cleavage of the 180 kDa chains. As shown in table 1, a quite linear relation between the extent of bait region cleavage and the chymotrypsin-α_2M ratio in the molar range 0–2 proteases per α_2M is found, i.e. two mol of chymotrypsin are necessary to cleave the four peptide chains of α_2M.

α_2M is now allowed to react with chymotrypsin in the presence of 40 mM hydroxylamine. This primary amine, at this concentration, does not inactivate α_2M and prevents covalent bond formation between the protease and the inhibitor [15] as does the commonly used methylamine [7]. Indeed, the faint bands disappear (fig.1). Furthermore, a single chymotrypsin molecule is then able to bring to completion the 180 kDa proteolysis (table 1).

In an attempt to correlate the extent of bait region cleavage with the binding mode of the protease to the inhibitor, different α_2M-chymotrypsin complexes were filtered under denaturing conditions. In amine-free buffer the yield of covalent binding is 95% and 85% for the 1:1 and 1:2 α_2M-protease complexes, respectively. In contrast,

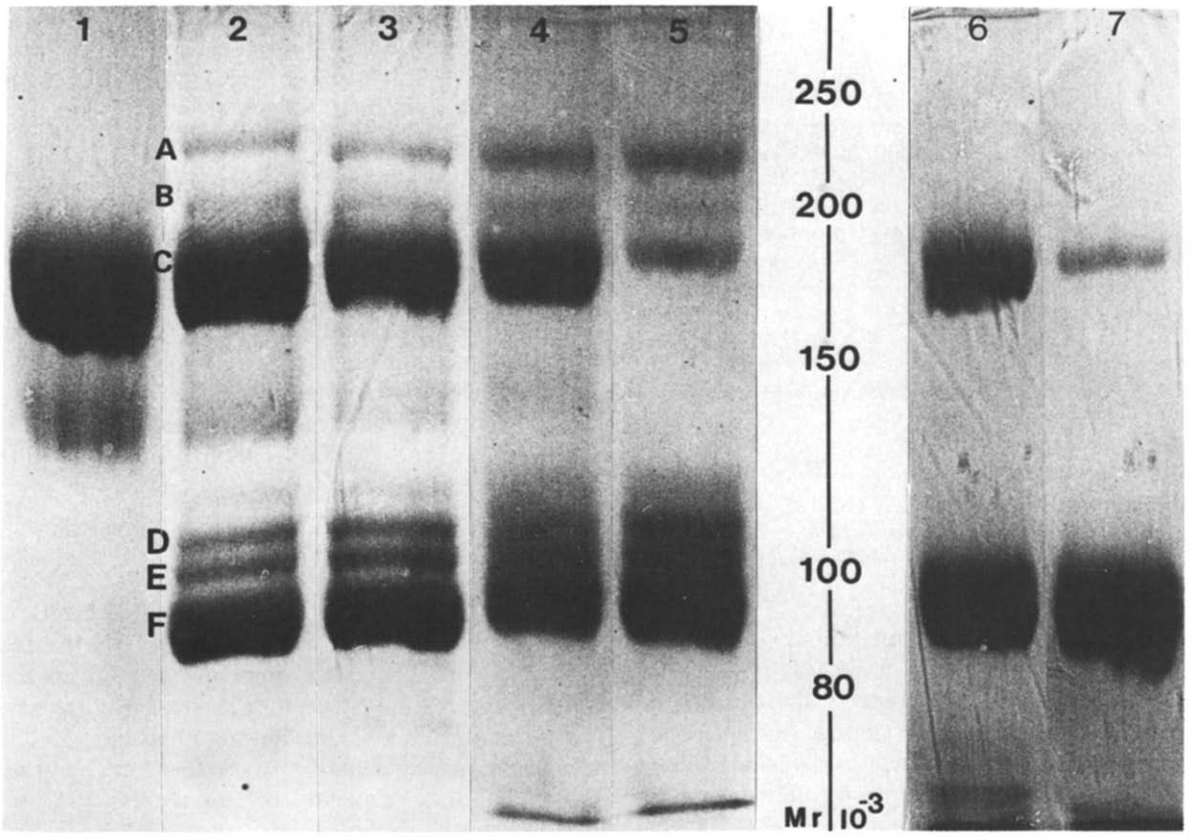

Fig.1. Polyacrylamide gel electrophoresis of chymotrypsin-α_2-macroglobulin conjugates under denaturing and reducing conditions. Lanes 1–5 show the pattern obtained with none (1), 0.5 (2), 1.0 (3), 1.5 (4) and 2.0 (5) equivalents of chymotrypsin per α_2M; lanes 6 and 7 show the effect of hydroxylamine (40 mM final concentration) on the chymotrypsin-α_2M interaction using molar ratios of 0.6 and 1.0, respectively.

covalent crosslinking is close to zero when the complexes are prepared in the presence of 40 mM hydroxylamine.

These data suggest that an intramolecular rather than an intermolecular proteolysis of α_2M by a non-covalently bound chymotrypsin molecule may be considered. We then investigated the behaviour of a non-covalent 1:1 chymotrypsin-α_2M complex in the presence of a large excess of native α_2M.

3.2. *The cleavage of the four α_2M peptide chains by a single non-covalently bound chymotrypsin proceeds by an intramolecular proteolysis*

In order to evidence that chymotrypsin, non-covalently bound to α_2M, is not able to proteolyse α_2M molecules other than those to which the protease is initially bound, experiments using energy transfer measurements were undertaken. For this purpose, FITC-labelled chymotrypsin (1 ± 0.1 label per chymotrypsin) is reacted with α_2M in the presence of 40 mM hydroxylamine and I-AEDns. The protease induces the appearance of the α_2M free thiol groups which then react with the sulfhydryl reagent [16]. The doubly labelled complex is then isolated by Sephadex G-25 filtration and the energy transfer efficiency value E determined from fluorescence quenching measurement of I-AEDns by FITC-labelled protease [11,16]. E is found to be 0.62. An aliquot is prepared in the same way in amine-free buffer and is used as a control.

Now, a 10-fold molar excess of native ^{125}I-α_2M is added and the mixture incubated at 37°C for

Table 1

Extent of bait region cleavage in chymotrypsin-[^{125}I]α_2-macroglobulin complexes

Chymotrypsin/α_2M (mol/mol)	% bait region cleavage[a]	
	In hydroxylamine free buffer	In the presence of 40 mM hydroxylamine
0.25	10	27
0.5	27	53
0.75	35	78
1.0	53	100
1.5	78	–
2.0	98	–

[a] The radioactivity recovered in each M_r 180000 and 90000 electrophoretic band was used to estimate the % of M_r 180000 disappearance; 100% = four bait regions per mol; the values are the average of four determinations

8 h. Every 30 min, aliquots are set apart for quenching and 90 kDa fragment appearance measurements. A displacement of the labelled protease from its initial complex by native α_2M to form a new complex should (i) decrease the transfer efficiency, the newly generated -SH groups of α_2M being not labelled, (ii) increase the 90 kDa fragment generation. On the other hand, an increase in the extent of 180 kDa chain cleavage without modification of the transfer efficiency value could result from proteolysis of α_2M subunits by α_2M-bound chymotrypsin.

It is observed that the initial value of E as well as the amount of 180 kDa chains are not significantly affected during the incubation time. However, a 10% decrease of the E value is observed and could be correlated to a further proteolysis of the initial complex, as suggested by the concomitant disappearance of 90 kDa fragments giving lower molecular mass products.

4. DISCUSSION

In the present study, the reaction of chymotrypsin with α_2M has been characterized by quantitating the extent of α_2M peptide chain cleavage with respect to the binding mode of the protease. The data describing the covalent binding mode of chymotrypsin with α_2M are consistent with a sequential mechanism in which each functional unit of the α_2M molecule is successively altered as already observed with other proteases [17–19]. But the non-covalent binding of chymotrypsin induces the modification of the two protease-binding sites of the inhibitor.

It can be pointed out that the non-covalently bound chymotrypsin molecule is always 'trapped' within the α_2M molecule as suggested by the following observations. Indeed, whatever the binding mode of chymotrypsin to α_2M (1 : 1 molar ratio), we found that (i) under the electron microscope all complexes display the same cyrillic J type Ж-shape, (ii) the catalytic properties of the bound proteases towards Suc-Ala$_2$-Pro-Phe-pNA are always lowered to about 65% with respect to the free enzyme, (iii) the values of fluorescence quenching (25%) and red shift (3 nm) of the FITC-labelled protease measured upon α_2M fixation are similar; furthermore, energy transfer efficiencies from the labelled -SH groups of α_2M towards the labelled protease are not significantly different, thus supporting the idea that the covalent and non-covalent protease-binding sites of α_2M have a quite similar localization in the complexes, (iv) the polarization values of the FITC-labelled chymotrypsin present the same variations upon α_2M fixation (p = 0.17 and 0.35 for the free and α_2M-bound enzyme).

In conclusion, the alteration of the two protease-binding sites of α_2M by a single molecule of chymotrypsin is compatible with the α_2M structure as observed by electron microscopy [12] and implies a close localization of the two protease-binding sites of α_2M [10] on each site of the binding surface of the α_2M subunits [20]. Our results also support a recently proposed model of the protease-α_2M complex [21] which suggests a partial occupancy by a single protease molecule of the two protease binding sites of α_2M.

REFERENCES

[1] Swenson, R.P. and Howard, J.B. (1979) J. Biol. Chem. 254, 4452–4456.
[2] Sottrup-Jensen, L., Stepanik, T.M., Kristensen, T., Wierzbicki, D.M., Jones, J.C., Lonblad, P.B., Magnusson, S. and Petersen, T.E. (1984) J. Biol. Chem. 259, 8318–8327.

[3] Hall, P.K. and Roberts, R.C. (1978) Biochem. J. 171, 27–38.
[4] Sottrup-Jensen, L., Petersen, T.E. and Magnusson, S. (1980) FEBS Lett. 121, 275–279.
[5] Howard, J.B. (1981) Proc. Natl. Acad. Sci. USA 78, 2235–2239.
[6] Barrett, A.J. and Starkey, P.M. (1973) Biochem. J. 133, 709–724.
[7] Sottrup-Jensen, L., Petersen, T.E. and Magnusson, S. (1981) FEBS Lett. 128, 123–126.
[8] Gonias, S.L. and Pizzo, S.V. (1983) Biochemistry 22, 536–546.
[9] Kurecki, T., Kress, L.F. and Laskowski, M. (1979) Anal. Biochem. 99, 415–420.
[10] Pochon, F., Favaudon, V., Tourbez-Perrin, M. and Bieth, J.G. (1981) J. Biol. Chem. 256, 547–550.
[11] Pochon, F., Steinbuch, M., Lambin, P. and Kichenin, V. (1983) FEBS Lett. 161, 51–54.
[12] Tapon-Bretaudière, J., Bros, A., Couture-Tosi, E. and Delain, E. (1985) EMBO J. 4, 85–89.
[13] Harpel, P.C. and Mosesson, M.W. (1973) J. Clin. Invest. 52, 2175–2184.
[14] Brown, J.R. and Hartley, B.J. (1966) Biochem. J. 101, 214–228.
[15] Lambin, P., Pochon, F., Fine, J.M. and Steinbuch, M. (1983) Thromb. Res. 32, 123–132.
[16] Pochon, F., Favaudon, V. and Bieth, J. (1983) Biochem. Biophys. Res. Commun. 111, 964–969.
[17] Ganrot, P.O. (1967) Acta Chem. Scand. 21, 602–608.
[18] Christensen, U. and Sottrup-Jensen, L. (1984) Biochemistry 23, 6619–6626.
[19] Steiner, J.P., Bhattacharya, P. and Strickland, D.K. (1985) Biochemistry 24, 2993–3001.
[20] Osterberg, R. and Pap, S. (1983) Ann. NY Acad. Sci. 421, 98–111.
[21] Feldman, S.R., Gonias, S.L. and Pizzo, S.V. (1985) Proc. Natl. Acad. Sci. USA 82, 5700–5704.