Structural arrangement in the α_2-macroglobulin–thrombin complex

François Pochon, Marion Steinbuch*, Patrick Lambin+ and Violaine Kichenin*

*Orsay, U.219 INSERM, Institut Curie, Biologie, 91405 – *CNTS, 91400 Orsay* and +*CNTS, Paris 15ème, France*

Received 20 June 1983

The cysteine sulfhydryl groups of α_2-macroglobulin (α_2M) generated upon thrombin complex formation are in contact with the proteinase surface as evidenced by singlet–singlet energy transfer measurements from *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid-labeled thiol functions of α_2M to fluorescein isothiocyanate-labeled thrombin. The thrombin-α_2M binding is normally covalent, but the presence of hydroxylamine during the reaction leads to the formation of a non-covalent complex. The transfer energy determinations show that the α_2M binding sites of thrombin are quite similar, whatever covalent or non-covalent binding occurs.

α_2-Macroglobulin *Thrombin* *Antithrombin* *Chymotrypsin*

1. INTRODUCTION

The α_2-macroglobulin (α_2M) molecule is composed of 2 subunits held together by non-covalent forces. Each of them contains 2 identical polypeptide chains linked by disulfide bridges. All 4 chains contain a reactive site identified as a thioester cross-link between a cysteine and a glutamic acid residue [1–4]. Binding of proteases, or treatment by primary amines, results in the appearance of 4 thiol groups and as many activated glutamic acid residues able to react covalently with amino groups [2,5,6]. The interaction of one α_2M molecule with 2 trypsins corresponds to covalent binding of 1.3 molecule, 2.2 ± 0.1 of the activated Glx residues being engaged in this linkage [7].

In [8], using singlet–singlet energy transfer measurements between labeled thiol functions of α_2M and labeled proteases, it was suggested that the chymotrypsin binding sites of α_2M were probably not too different whatever covalent or non-covalent binding was concerned. However, a definite answer could not be given, the two modes of binding occurring during the protease–α_2M complex formation as described for trypsin.

Here, this hypothesis was reinvestigated by the same experimental approach, a simple method allowing to obtain specifically covalent or non-covalent binding of thrombin by α_2M.

Abbreviations: α_2M, α_2-macroglobulin; FITC, fluorescein isothiocyanate; IAEDns, *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonate

2. EXPERIMENTAL

2.1. *Materials*

Human α_2M was prepared by Zn^{2+} affinity chromatography [9] from fresh plasma and was 85% active as tested by trypsin binding [10]. Thrombin was obtained as in [11], the enzyme showed a specific activity of 2500 NIH units/mg. Anti-thrombin III (AT III) was prepared by affinity chromatography using fraction IV as starting material and had a specific activity of 5.3 units/mg [11].

2.2. *Determination of enzymatic activities*

Protease activities were evaluated by the amidolytic activity monitoring on a Cary 118 C at 37°C; the hydrolysis rates of chromogenic substrates at 405 nm were Suc–Ala_2–Pro–Phe-

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

pNA from Bachem (USA) and H D Phe–Pip–Arg-pNA from Kabi Diagnostica (Sweden) being used for chymotrypsin and thrombin, respectively. In the presence of heparin, polybrene at a final concentration of 1 mg/ml was added to the reaction mixture according to the manufacturer's instructions.

2.3. *Labeling of proteases*

Chymotrypsin and thrombin were FITC-labeled as in [10,11]. The extent of labeling was determined from the ratio of the dye absorption at 495 nm (E = 66.8 $mM^{-1}.cm^{-1}$) to that of the enzymes at 280 nm (E = 45 and 61 $mM^{-1}.cm^{-1}$, respectively).

2.4. *Preparation of labeled and non-labeled protease–α_2M complexes*

All complexes were prepared in 0.1 M NaCl, 50 mM Tris–HCl (pH 7.5) and purified by Sephacryl S 200 filtration in this buffer.

2.4.1. α_2M–Thrombin complexes

Labeled or unlabeled thrombin was added to 1 μM solution of α_2M in the presence or absence of the specific SH reagent IAEDns [12] (final concentration 1×10^{-4} M). The reaction could be performed in the presence of 0.1 M hydroxylamine [13]. The reaction mixtures were left 10 min at room temperature and the complexes were isolated as indicated.

2.4.2. Chymotrypsin–α_2M–thrombin complexes

This asymmetric complex was prepared in the same way: to 2.7 μM α_2M (in presence or absence of IAEDns) were first added 3 μM thrombin followed 1 min later by 3 μM chymotrypsin, the proteases being FITC-labeled or not. The extent of protease fixation was estimated using the indicated specific chromogenic substrates.

2.5. *Estimation of covalently bound thrombin*

Complexes of α_2M with FITC-labeled thrombin were dialyzed against 6 M guanidine chloride, 0.5 M Tris–HCl (pH 8.0) buffer and filtered on Sephacryl S 300 in the same buffer. The extent of covalent binding of thrombin to α_2M was estimated from the fluorescence peak area corresponding to FITC-labeled thrombin co-migrating with high-M_r fragments of α_2M.

2.6. *Energy transfer measurements*

Transfer efficiencies were determined from the fluorescence quenching of IAEDns–α_2M by the bound FITC-labeled protease, by excitation at 360 nm and emission at 470 nm. The average transfer efficiencies were given by the relation $e = 1 - F_1/F_2$, where F_1 and F_2 were the normalized fluorescence intensities of the donor (IAEDns) in the presence and in absence of the acceptor (FITC), respectively. A modified [10] Farrand Mark I was used for fluorescence determinations.

3. RESULTS AND DISCUSSION

3.1. *General characterization of the α_2M–thrombin complexes*

The free α_2M sulfhydryl groups which appeared upon thrombin-induced thioester cleavage, react with the specific -SH reagent IAEDns to an extent of 4 ± 0.2 labels per α_2M molecule as estimated from the dye absorption at 335 nm of the isolated complex. On the other hand, the stoichiometry of the FITC–thrombin–α_2M complex is 0.92. Gel filtration of this complex under denaturing conditions shows that covalent binding of the protease occurs to an extent of 95 ± 5%. The presence of 2.3 FITC labels per thrombin molecule does not modify the rate of synthetic substrate hydrolysis but this rate is decreased to about 48% by linkage of the protease to α_2M.

The thrombin–α_2M stoichiometry is not greatly modified by the presence of hydroxylamine during the complex formation, it is found to be 0.80, but the α_2M-binding of the protease becomes 95 ± 5% non-covalent. In this complex, the thrombin activity on the synthetic substrate is decreased to about 58% with respect to the free enzyme.

A 2000-fold molar excess of anti-thrombin III, with or without heparin, is unable to inhibit the activity of the complexed thrombin upon synthetic substrate, whatever the mode of binding, and even after 24 h of incubation at 37°C. These results show that complexed thrombin cannot react with its specific inhibitor and that non-covalently bound thrombin cannot be displaced as easily from its complex. The situation is therefore different from that observed with the α_2M–trypsin complex where inhibition [14] and displacement [15] of trypsin was observed after addition of soybean trypsin inhibitor.

Thrombin–α_2M–chymotrypsin complex can be easily obtained by sequential addition of thrombin and chymotrypsin to α_2M. The extent of enzyme fixation may be determined by estimation of the activities of the proteases for their specific substrates, the rates of peptide hydrolysis being corrected for proteases–α_2M binding (+12% and −50% for chymotrypsin and thrombin, respectively). Under the experimental conditions already described, 1.05 ± 0.1 thrombin and 0.9 ± 0.1 chymotrypsin molecules are bound per α_2M molecule.

3.2. *Energy transfer measurements*

Singlet–singlet energy transfer efficiencies were measured from fluorescence quenching of IAEDns labels bound to protease-induced thiol groups of α_2M by FITC-labeled proteases. Complexes of IAEDns–α_2M with unlabeled proteases and inversely unlabeled α_2M with FITC proteases were also isolated and served as controls in absence of IAEDns to FITC transfer. In order to interpret the transfer efficiencies in terms of distance between donor and acceptor molecules, we use the model in [16]; i.e., we assume:

(i) A random distribution of the labels on the chymotrypsin and thrombin surface, these enzymes being considered as rigid spheres of a hydrous radius r = 20 and 23 Å, respectively, as computed from molecular mass and partial specific volume (0.735 cm^3/g) values;
(ii) The probability of finding labels obeys a Poisson distribution;
(iii) A mean value of the dipole–dipole orientation factor $K^2 = 0.67$;
(iv) A characteristic transfer distance between IAEDns and FITC R_o = 44 Å [17].

The transfer efficiency value (table 1) determined for the asymmetrical chymotrypsin–α_2M–thrombin complex is very close to that calculated for a single sphere transfer model; i.e., for an individual protease surface labeled with both donor and acceptor molecules as previously described for the symmetrical chymotrypsin–α_2M–chymotrypsin complex. The donor-labeled -SH groups of α_2M are in close contact with both chymotrypsin and thrombin surface.

On the other hand, the *e*-value within the covalent thrombin–α_2M complex is significantly lower than that expected from the thrombin radius and the average labeling of the protease. This may result from the fact that energy transfer occurs within one α_2M subunit, a cross transfer from donor molecules of one α_2M subunit to acceptor molecules of the protease located on the other subunit being very unlikely despite the proximity of the two protease binding sites [10].

Lastly, non-covalent binding of thrombin decreases the energy transfer efficiency to about 15–20% indicating that the α_2M–thrombin interaction is only slightly modified. This difference could be the consequence of a small displacement of the non-covalent bound thrombin molecule in its specific α_2M binding site as also suggested by the difference in the hydrolysis rate of the synthetic substrate. However, there is no drastic change in the environment of the bound enzyme. Covalently and non-covalently bound thrombin molecules are therefore located at identical sites in the α_2M–thrombin complex. This strengthens the view that covalent bonds do not contribute significantly to the protease–α_2M binding energy [18].

It has been shown [13] that thrombin (even in excess) cleaves only 2 chains of the α_2M molecule as has recently been reported for the plasmin α_2M in-

Table 1

Stoichiometry, average number of bound labels per α_2M and α_2M bound protease and energy transfer efficiencies, *e*, in different α_2M–protease complexes

Complex	IAEDns (α_2M)	FITC (protease)	*e*
Chymotrypsin–α_2M–thrombin (1:1:1)	4	2.5 and 2.3	0.85
Thrombin (covalent)–α_2M (1:1)	4	2.3	0.65
Thrombin (non-covalent)–α_2M (1:1)	4	2.3	0.48

teraction [19]. However, all α_2M molecules are converted to the fast form by thrombin. The fast α_2M–thrombin complex can react with chymotrypsin demonstrating the accessibility of the reaction sites. The remaining 2 chains are normally cleaved. This observation is similar to the recently reported interaction of trypsin with the α_2M–plasmin complex [19]. Whereas the non-covalent thrombin–α_2M complex does not resist to denaturing aspects (SDS, guanidine), its stability in the presence of AT III and heparin is remarkable.

ACKNOWLEDGEMENT

We thank Dr J. Bieth for helpful discussions.

REFERENCES

[1] Swenson, R.P. and Howard, J.B. (1979) Proc. Natl. Acad. Sci. USA 76, 4313–4316.
[2] Sottrup-Jensen, L., Petersen, T.E. and Magnusson, S. (1980) FEBS Lett. 121, 275–279.
[3] Sottrup-Jensen, L., Hansen, H.F., Mortensen, S.B., Petersen, T.E. and Magnusson, S. (1981) FEBS Lett. 123, 145–148.
[4] Howard, J.B. (1981) Proc. Natl. Acad. Sci. USA 78, 2235–2239.
[5] Sottrup-Jensen, L., Petersen, T.E. and Magnusson, S. (1981) FEBS Lett. 128, 123–126.
[6] Sottrup-Jensen, L., Petersen, T.E. and Magnusson, S. (1981) FEBS Lett. 128, 127–132.
[7] Sottrup-Jensen, L. and Hansen, H.F. (1982) Biochem. Biophys. Res. Commun. 107, 93–100.
[8] Pochon, F., Favaudon, V. and Bieth, J. (1983) Biochem. Biophys. Res. Commun. 111, 964–969.
[9] Kurecki, T., Kress, L.F. and Laskowski, M. sr (1979) Anal. Biochem. 99, 414–420.
[10] Pochon, F., Favaudon, V., Tourbez-Perrin, M. and Bieth, J. (1981) J. Biol. Chem. 256, 547–550.
[11] Pochon, F., Lambin, P. and Steinbuch, M. (1982) Thromb. Res. 26, 307–317.
[12] Hudson, E.N. and Weber, G. (1973) Biochemistry 12, 4154–4161.
[13] Lambin, P., Pochon, F., Fine, J.M. and Steinbuch, M. (1983) Thromb. Res., in press.
[14] Bieth, J., Tourbez-Perrin, M. and Pochon, F. (1981) J. Biol. Chem. 256, 7954–7957.
[15] Wu, K., Wang, D. and Feinman, R.D. (1981) J. Biol. Chem. 256, 10409–10414.
[16] Gennis, R.B. and Cantor, C.R. (1972) Biochemistry 11, 2509–2517.
[17] Huang, K.H., Fairclough, R.H. and Cantor, C.R. (1975) J. Mol. Biol. 97, 443–470.
[18] Salvesen, G.S. and Barret, A.J. (1980) Biochem. J. 187, 695–701.
[19] Howell, J.B., Beck, T., Bates, B. and Hunter, M.J. (1983) Arch. Biochem. Biophys. 221, 261–270.