

# Rat plasma $\alpha_1$ -inhibitor<sub>3</sub>: a member of the $\alpha$ -macroglobulin family

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The overall mechanism of interaction with proteinases of  $\alpha_1$ -inhibitor<sub>3</sub>, a plasma proteinase inhibitor so far specific to the rat, has been shown to be closely similar to that described for  $\alpha$ -macroglobulins. This mechanism includes: (i) the cleavage of at least one susceptible peptidic bond which leads to structural changes in the molecule. (ii) The cleavage of a putative thiol ester bond in another site of the molecule which permits the covalent linkage of the enzyme. Moreover, fragmentation of  $\alpha_1$ -inhibitor<sub>3</sub> upon heating as observed for  $\alpha$ -macroglobulin quarter subunits has been demonstrated. The question is raised of the presence of such a molecule in rat plasma in addition to two  $\alpha$ -macroglobulin species, all of these proteinase inhibitors being antigenically unrelated.

$\alpha_1$ -Inhibitor<sub>3</sub>       $\alpha$ -Macroglobulin      Rat plasma      Thiol ester      Heat fragmentation

## 1. INTRODUCTION

$\alpha$ -Macroglobulins have the unique property of giving enzymatically active complexes when they interact with proteinases (recent review [1]). In rat plasma, however, in addition to  $\alpha_1$ -macroglobulin and  $\alpha_2$  acute phase macroglobulin, a third protein, namely  $\alpha_1$ I<sub>3</sub>, is also able to give such active complexes when binding serine or cysteine proteinases [2,3]. Circulating  $\alpha_1$ I<sub>3</sub> has an  $M_r$  next to that of the quarter subunit of either human  $\alpha_2$ M or rat  $\alpha_2$ APM as well as the higher subunit of rat  $\alpha_1$ M [4,5]. It stands in normal rat plasma at a relatively high concentration (about 30  $\mu$ M) but behaves as a negative acute phase reactant [2]. As found for  $\alpha$ M, a conformational change of the  $\alpha_1$ I<sub>3</sub> molecule

is associated with the formation of a complex with proteinases [6].

Evidence is given here that the mechanism of interaction of  $\alpha_1$ I<sub>3</sub> with proteinases closely resembles that described for  $\alpha$ M. In particular, the appearance of a free thiol group presumably issuing from the cleavage of a thiol ester bond upon interaction with proteinases or methylamine has been demonstrated as well as a selective heat fragmentation of the molecule which is prevented by prior incorporation of methylamine. This structural relationship between  $\alpha_1$ I<sub>3</sub> and the quarter subunit of  $\alpha$ M raises the question of the simultaneous presence of these proteins in rat plasma and that of the phylogeny of these molecules.

## 2. MATERIALS AND METHODS

Rat  $\alpha_1$ I<sub>3</sub> was prepared from citrated rat plasma as in [3]. Human  $\alpha_2$ -macroglobulin was prepared using the method of Virca et al. [7]. Proteins were concentrated and equilibrated in 0.05 M Tris-HCl buffer (pH 7.4), 0.15 M NaCl. Their concentration was determined using an  $E_{280\text{nm}, 1\text{cm}}^{1\%}$  of 7.5 for  $\alpha_1$ I<sub>3</sub>

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**Abbreviations:**  $\alpha_1$ I<sub>3</sub>,  $\alpha_1$ -inhibitor<sub>3</sub>;  $\alpha$ M,  $\alpha$ -macroglobulin;  $\alpha_2$ APM,  $\alpha_2$  acute phase macroglobulin; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; Nbs, 3-carboxy-4-nitrobenzenethiol; E<sub>64</sub>, L-3-carboxy-*trans*-2,3-epoxypropionylleucylamido-(4-guanidino)butane

[3] and 9.1 for  $\alpha_2\text{M}$  [8]. Methylamine was obtained from Merck-Schuchardt, DTNB and iodoacetamide were from Sigma. Bovine chymotrypsin and papain were purchased from Boehringer. Chymotrypsin was prepared in 0.05 M Tris-HCl buffer (pH 7.4), 0.15 M NaCl, 0.02 M  $\text{CaCl}_2$  and papain as described elsewhere [9]. The cysteine proteinase inhibitor  $\text{E}_{64}$  was supplied by the Peptide Research Foundation (Osaka, Japan) and was dissolved in dimethyl sulfoxide to make a 1 mM stock solution in water.

All absorbance measurements were performed with a Jobin Yvon JY 201 spectrophotometer. Liberation of Nbs was determined from measurements at 412 nm using a molar absorption coefficient of  $13600 \text{ M}^{-1} \cdot \text{cm}^{-1}$  at pH 8.0 [10].

Slab gel electrophoresis in the presence of SDS was performed as described by O'Farrell [11] either with a 7.5% polyacrylamide gel or a 5–20% gradient gel. Samples were treated with 0.062 M Tris-HCl (pH 6.8), 0.1% SDS, 1% mercaptoethanol. Gels were run at 7 mA overnight and stained with Coomassie brilliant blue G in 12.5% trichloroacetic acid.

### 3. RESULTS AND DISCUSSION

Purified  $\alpha_1\text{I}_3$  samples incubated for 5 min at  $90^\circ\text{C}$  in 0.1% SDS, 1% 2-mercaptoethanol give a single band of  $M_r 200000 \pm 5000$  after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a Sigma calibration kit. When the incubation is made at  $90^\circ\text{C}$  for 160 min without reducing agent, then 2-mercaptoethanol is added for 5 min as before, two bands of  $M_r 147000$  and  $65000$  are generated. These fragments are clearly distinguishable from those obtained after incubation of  $\alpha_1\text{I}_3$  with proteinases such as  $\alpha$  chymotrypsin as shown in fig.1.

Such a heat dependent fragmentation appears as a common feature of  $\alpha_1\text{I}_3$  and  $\alpha\text{M}$  either from human or rat plasma [4,12] whose quarter subunits are cleaved into two fragments of different size. The mechanism responsible for  $\alpha\text{M}$  fragmentation upon heating has been shown to involve the cleavage of both a thioester bond and an adjacent peptidic bond [13].

Probably the same mechanism occurs in  $\alpha_1\text{I}_3$ , as suggested by the demonstration of the exposure of a thiol group on  $\alpha_1\text{I}_3$  upon incubation with a

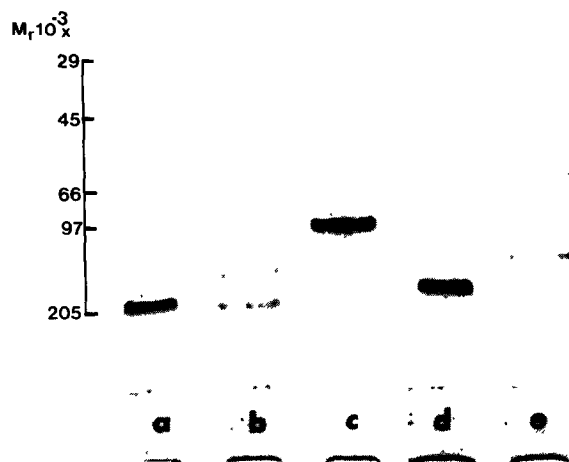


Fig.1. SDS-PAGE of (a)  $9 \mu\text{g}$  of 2-mercaptoethanol reduced  $\alpha_1\text{I}_3$ ; (b)  $18 \mu\text{g}$  of 2-mercaptoethanol reduced  $\alpha_1\text{I}_3$  first incubated for 160 min at  $90^\circ\text{C}$ ; (c)  $13 \mu\text{g}$  of reduced  $\alpha_1\text{I}_3$  previously incubated with a slight molar excess of chymotrypsin; (d)  $7 \mu\text{g}$  of human  $\alpha_2\text{M}$  treated as in (a); (e)  $21 \mu\text{g}$  of human  $\alpha_2\text{M}$  treated as in (b).  $M_r$  markers are from Sigma.

nucleophile as methylamine. Such an interaction prevents  $\alpha_1\text{I}_3$  from heat fragmentation, as has also been demonstrated for  $\alpha\text{M}$  [13]. Fig.2 shows a replot of the time dependent liberation of thiol groups from  $\alpha_1\text{I}_3$  upon incubation with methylamine. The reaction was followed by the increase in absorbance at 412 nm due to the liberation of Nbs from DTNB [10] and was shown to be pseudo-first-order under the conditions used. A maximal release of 1 SH per mol  $\alpha_1\text{I}_3$  was achieved after about 15 min. Determination of the pseudo-first-order rate constant from the slope of the curve in fig.2 allows the calculation of the second-order rate constant for the appearance of thiol groups on  $\alpha_1\text{I}_3$  using a  $\text{p}K_a$  of 10.4 for methylamine to determine the concentration of the unprotonated amine, which has been previously shown to be the active species in the reaction with human  $\alpha_2\text{M}$  [14], possibly due to the hydrophobic

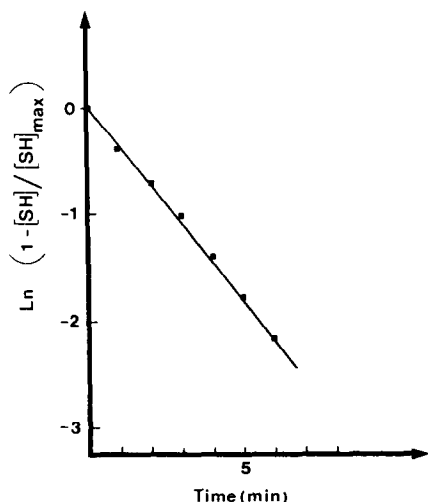


Fig.2. First-order plot of the time course of SH appearance on  $\alpha_1I_3$  upon methylamine treatment. The reaction was followed using DTNB and measuring the increase in absorbance at 412 nm. 200  $\mu$ l of  $\alpha_1I_3$  ( $6 \times 10^{-5}$  M) were diluted in 500  $\mu$ l of 0.04 M Tris-HCl buffer (pH 8.0), containing 50  $\mu$ l of DTNB (2.56 mM) then mixed with 50  $\mu$ l of 0.40 M methylamine in 0.05 M Tris-HCl buffer (pH 8.0).

environment required to maintain the stability of the thioester [15]:

$$k_2 = \frac{k(\text{pseudo } 1)}{[\text{CH}_3\text{NH}_2]}$$

with

$$k(\text{pseudo } 1) = \ln\left(1 - \frac{SH}{SH_{\max}}\right)$$

A value of  $58.3 \text{ M}^{-1} \cdot \text{s}^{-1}$  at pH 8.0 was found for the second order rate constant, which means that the reaction of methylamine with  $\alpha_1I_3$  proceeds at an about 5-fold faster rate than that of methylamine with human  $\alpha_2M$  under the same conditions [14,16]. Addition of iodoacetamide (15 mM) in the same  $\alpha_1I_3$  methylamine mixture as before and further incubation for 10 min at  $37^\circ\text{C}$  leads to complete inhibition of the increase in  $A_{412\text{nm}}$  upon addition of DTNB. This therefore gives evidence that the group exposed by incubation of  $\alpha_1I_3$  and methylamine and made available to titration by DTNB is a thiol group.

When  $\alpha_1I_3$  is incubated with a slight molar excess of chymotrypsin instead of methylamine, and DTNB is added to the mixture, an increase in absorbance at 412 nm is also observed. However, the reaction occurs much more rapidly than before so that a titration curve of exposed SH by chymotrypsin can be obtained. 6.6  $\mu$ mol SH appear per 7.1  $\mu$ mol  $\alpha_1I_3$  after at least 7.5  $\mu$ mol chymotrypsin have been reacted (fig.3). It may be thus concluded that one mol chymotrypsin interacts with one mol  $\alpha_1I_3$  and generates one SH per mol of inhibitor. This confirms previous results obtained for the determination of the  $\alpha_1I_3$  chymotrypsin stoichiometric ratio [6].

SDS-PAGE of  $\alpha_1I_3$  chymotrypsin mixture demonstrates two major bands of  $M_r$  112000 and 96000 (fig.4). However, the one with the higher  $M_r$  is further hydrolysed and rapidly disappears as the chymotrypsin concentration increases. This possibly depends on the proteolytic activity of  $\alpha_1I_3$  bound chymotrypsin as shown before [6]. Using papain, two major bands of  $M_r$  almost identical to those found for chymotrypsin ( $M_r$  110000 and 102000) are also observed, provided the reaction is rapidly blocked by addition of the irreversible cysteine proteinase inhibitor E64 (not shown).

From these results it may be concluded that the reactive center of  $\alpha_1I_3$  closely resembles that of  $\alpha M$

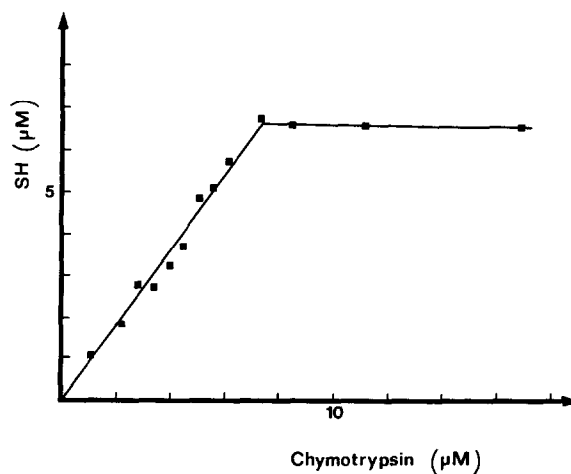


Fig.3. Titration of thiol-group appearance upon addition of chymotrypsin to  $\alpha_1I_3$ . 70  $\mu$ l of  $\alpha_1I_3$  ( $5.3 \times 10^{-5}$  M) were mixed with 400–430  $\mu$ l of 0.04 M Tris-HCl buffer (pH 8.0) containing 50  $\mu$ l of DTNB (2.56 mM) and 0–30  $\mu$ l of chymotrypsin ( $4.3 \times 10^{-4}$  M).  $A_{412\text{nm}}$  is recorded after 1 min.

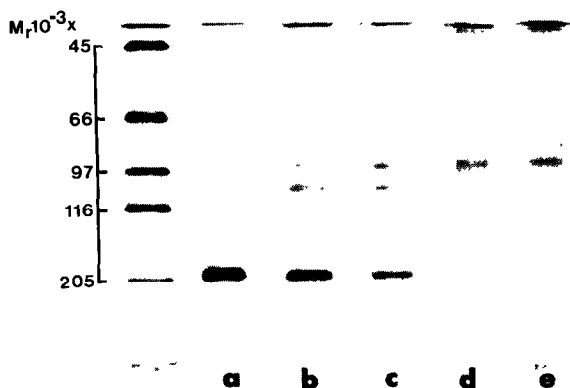


Fig.4. SDS-PAGE of reduced  $\alpha_1\text{I}_3$  (9  $\mu\text{g}$ ) incubated for 2 min at 37°C with increasing amounts of chymotrypsin.  $\alpha_1\text{I}_3$ /chymotrypsin molar ratios were (a) 1:0; (b) 1:0.25; (c) 1:0.5; (d) 1:1; (e) 1:2.

since both include a thiolester bond which is cleaved by heat and denaturants and also by interaction with proteinases. As a result of the thiolester hydrolysis, proteinases may be covalently linked to the inhibitor and may remain active provided the reaction with their substrate is not sterically hindered [17].

The physical trap hypothesis [18], which may explain that proteinases are not always covalently linked to  $\alpha\text{M}$ , does not apply to  $\alpha_1\text{I}_3$  since the active site of  $\alpha_1\text{I}_3$  bound proteinases is still accessible to inhibitors of large  $M_r$  [6]. This could possibly be due to the monomeric structure of  $\alpha_1\text{I}_3$ . A proposal has been made [19] that the non-covalent binding mode of proteinases by  $\alpha\text{M}$  is an evolutionary step related to the selection of polymeric structures but this has been also refuted by others [20]. It therefore remains to be seen whether  $\alpha_1\text{I}_3$  represents a primitive form of one or both  $\alpha\text{M}$  species present in the rat or whether all of these molecules have evolved from a common ancestor.

To the structural identity between the reactive sites of  $\alpha_1\text{I}_3$  and the quarter  $\alpha\text{M}$  subunit may be added a functional identity since  $\alpha_1\text{I}_3$  proteinase complexes, like those involving  $\alpha\text{M}$ , are rapidly eliminated from the circulation. The half-life of  $\alpha_1\text{I}_3$  proteinase complexes has been previously found to be about 15 min [21] whereas nascent  $\alpha_1\text{I}_3$  is eliminated with a half-life of  $51 \pm 2$  h (unpublished). This is suggestive of a specific membrane receptor to proteinase modified  $\alpha_1\text{I}_3$ , as previously found for  $\alpha\text{M}$ . The possible identity of

these receptors would undoubtedly provide an additional proof to the homology of these inhibitors. However, a final answer will be given only when the primary structure, of rat  $\alpha_1\text{M}$  and  $\alpha_2\text{M}$  subunits as well as that of  $\alpha_1\text{I}_3$  are known. It is worth noting that no common antigenic property has been found between these three molecules. Neither was any relationship between rat  $\alpha_1\text{I}_3$  and the other reactive thiolester containing proteins present in plasma demonstrated, i.e., complement C<sub>3</sub> and C<sub>4</sub> [22,23]. This means that probably at least 5 proteins of rat plasma include an internal thiolester in their molecular structure.

On the other hand,  $\alpha_1\text{I}_3$  is a typical negative acute phase reactant, the concentration of which decreases down to 30% of normal after turpentine injection [2] whereas at the same time one of the two rat  $\alpha\text{M}$  species, i.e.,  $\alpha_2\text{APM}$  increases dramatically [24]. Whether or not both phenomena are related still remains to be elucidated.

Also puzzling is the fact that  $\alpha_1\text{I}_3$  has been so far described only in rat plasma. Whether or not this molecule has disappeared from other species during evolution or has diverged from an  $\alpha\text{M}$  ancestor in the rat is a point which has to be clarified, probably in connection with the presence of two different  $\alpha\text{M}$  varieties in several mammal species [25].

## REFERENCES

- [1] Harpel, P.C. and Brower, M.S. (1983) *Ann. NY Acad. Sci.* 421, 1–9.
- [2] Gauthier, F. and Ohlsson, K. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* 359, 987–992.
- [3] Esnard, F. and Gauthier, F. (1980) *Biochim. Biophys. Acta* 614, 553–563.
- [4] Nelles, L.P. and Schnebli, H.P. (1982) *Hoppe-Seyler's Z. Physiol. Chem.* 363, 677–682.
- [5] Schaeufele, J.T. and Koo, P.H. (1982) *Biochem. Biophys. Res. Commun.* 108, 1–7.
- [6] Esnard, F., Gauthier, F. and Maurizot, J.C. (1981) *Biochimie* 63, 767–774.
- [7] Virca, G.D., Travis, J., Hall, P.K. and Roberts, R.C. (1978) *Anal. Biochem.* 89, 274–278.
- [8] Dunn, J.T. and Spiro, R.G. (1967) *J. Biol. Chem.* 242, 4007–4021.
- [9] Esnard, F. and Gauthier, F. (1983) *J. Biol. Chem.* 258, 12443–12447.
- [10] Silverstein, R.M. (1975) *Anal. Biochem.* 63, 281–282.

- [11] O'Farrell, P.H. (1975) *J. Biol. Chem.* 250, 5549–5555.
- [12] Harpel, P.C., Hayes, M.B. and Hugli, T.E. (1979) *J. Biol. Chem.* 254, 8669–8678.
- [13] Howard, J.B. (1981) *Proc. Natl. Acad. Sci. USA* 78, 2235–2239.
- [14] Larsson, L.J. and Bjork, I. (1984) *Biochemistry* 23, 2802–2807.
- [15] Van Leuven, F. (1982) *Trends Biochem. Sci.* 7, 185–187.
- [16] Strickland, D.K., Bhattacharya, P. and Olson, S.T. (1984) *Biochemistry* 23, 3115–3124.
- [17] Sottrup-Jensen, L., Petersen, T.E. and Magnusson, S. (1981) *FEBS Lett.* 128, 127–132.
- [18] Barrett, A.J. and Starkey, P.M. (1973) *Biochem. J.* 133, 709–724.
- [19] Feinman, R.D. (1983) *Ann. NY Acad. Sci.* 421, 472–476.
- [20] Starkey, P.M. and Barrett, A.J. (1982) *Biochem. J.* 205, 105–115.
- [21] Gauthier, F., Genell, S., Mouray, H. and Ohlsson, K. (1979) *Biochim. Biophys. Acta* 566, 200–210.
- [22] Daha, M.R., Stufers-Heiman, M., Kijlstra, A. and Vanes, L.A. (1979) *Immunology* 36, 63–70.
- [23] Daha, M.R. and Vanes, L.A. (1980) *Immunobiology* 158, 72–75.
- [24] Gauthier, F. and Mouray, H. (1976) *Biochem. J.* 159, 661–665.
- [25] Starkey, P.M. and Barrett, A.J. (1977) in: *Proteinases in Mammalian Cells and Tissues* (Barrett, A.J. ed.) pp.663–696, Elsevier, Amsterdam, New York.