Rat plasma α_1-inhibitor$_3$: a member of the α-macroglobulin family

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The overall mechanism of interaction with proteinases of α_1-inhibitor$_3$, a plasma proteinase inhibitor so far specific to the rat, has been shown to be closely similar to that described for α-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 α_1-inhibitor$_3$ upon heating as observed for α-macroglobulin quarter subunits has been demonstrated. The question is raised of the presence of such a molecule in rat plasma in addition to two α-macroglobulin species, all of these proteinase inhibitors being antigenically unrelated.

α_1-Inhibitor$_3$ *α-Macroglobulin* *Rat plasma* *Thiol ester* *Heat fragmentation*

1. INTRODUCTION

α-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 α_1-macroglobulin and α_2 acute phase macroglobulin, a third protein, namely α_1I_3, is also able to give such active complexes when binding serine or cysteine proteinases [2,3]. Circulating α_1I_3 has an M_r next to that of the quarter subunit of either human α_2M or rat α_2APM as well as the higher subunit of rat α_1M [4,5]. It stands in normal rat plasma at a relatively high concentration (about 30 μM) but behaves as a negative acute phase reactant [2]. As found for αM, a conformational change of the α_1I_3 molecule is associated with the formation of a complex with proteinases [6].

Evidence is given here that the mechanism of interaction of α_1I_3 with proteinases closely resembles that described for αM. 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 α_1I_3 and the quarter subunit of αM 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 α_1I_3 was prepared from citrated rat plasma as in [3]. Human α_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 $E^{1\%}_{280\,nm,1\,cm}$ of 7.5 for α_1I_3

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Abbreviations: α_1I_3, α_1-inhibitor$_3$; αM, α-macroglobulin; α_2APM, α_2 acute phase macroglobulin; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; Nbs, 3-carboxy-4-nitrobenzenethiol; E_{64}, L-3-carboxy-*trans*-2,3-epoxypropionylleucylamido-(4-guanidino)butane

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[3] and 9.1 for α_2M [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 $CaCl_2$ and papain as described elsewhere [9]. The cysteine proteinase inhibitor 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 $M^{-1} \cdot cm^{-1}$ 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 α_1I_3 samples incubated for 5 min at 90°C in 0.1% SDS, 1% 2-mercaptoethanol give a single band of M_r 200000 ± 5000 after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a Sigma calibration kit. When the incubation is made at 90°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 α_1I_3 with proteinases such as α chymotrypsin as shown in fig.1.

Such a heat dependent fragmentation appears as a common feature of α_1I_3 and αM either from human or rat plasma [4,12] whose quarter subunits are cleaved into two fragments of different size. The mechanism responsible for αM 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 α_1I_3, as suggested by the demonstration of the exposure of a thiol group on α_1I_3 upon incubation with a

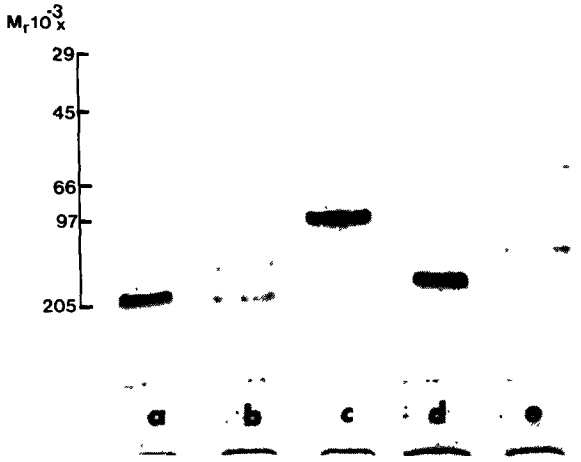

Fig.1. SDS–PAGE of (a) 9 μg of 2-mercaptoethanol reduced α_1I_3; (b) 18 μg of 2-mercaptoethanol reduced α_1I_3 first incubated for 160 min at 90°C; (c) 13 μg of reduced α_1I_3 previously incubated with a slight molar excess of chymotrypsin; (d) 7 μg of human α_2M treated as in (a); (e) 21 μg of human α_2M treated as in (b). M_r markers are from Sigma.

nucleophile as methylamine. Such an interaction prevents α_1I_3 from heat fragmentation, as has also been demonstrated for αM [13]. Fig.2 shows a replot of the time dependent liberation of thiol groups from α_1I_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 α_1I_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 α_1I_3 using a pK_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 α_2M [14], possibly due to the hydrophobic

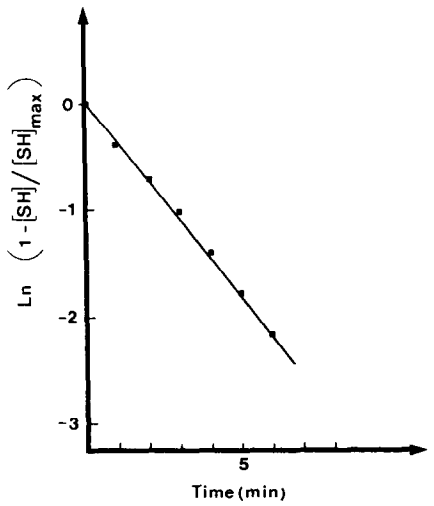

Fig.2. First-order plot of the time course of SH appearance on α_1I_3 upon methylamine treatment. The reaction was followed using DTNB and measuring the increase in absorbance at 412 nm. 200 μl of α_1I_3 (6×10^{-5} M) were diluted in 500 μl of 0.04 M Tris–HCl buffer (pH 8.0), containing 50 μl of DTNB (2.56 mM) then mixed with 50 μl 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]:

$$k_2 = \frac{k(\text{pseudo 1})}{[CH_3NH_2]}$$

with

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

A value of 58.3 $M^{-1} \cdot s^{-1}$ at pH 8.0 was found for the second order rate constant, which means that the reaction of methylamine with α_1I_3 proceeds at an about 5-fold faster rate than that of methylamine with human α_2M under the same conditions [14,16]. Addition of iodoacetamide (15 mM) in the same α_1I_3 methylamine mixture as before and further incubation for 10 min at 37°C leads to complete inhibition of the increase in A_{412nm} upon addition of DTNB. This therefore gives evidence that the group exposed by incubation of α_1I_3 and methylamine and made available to titration by DTNB is a thiol group.

When α_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 μmol SH appear per 7.1 μmol α_1I_3 after at least 7.5 μmol chymotrypsin have been reacted (fig.3). It may be thus concluded that one mol chymotrypsin interacts with one mol α_1I_3 and generates one SH per mol of inhibitor. This confirms previous results obtained for the determination of the α_1I_3 chymotrypsin stoichiometric ratio [6].

SDS–PAGE of α_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 α_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 α_1I_3 closely resembles that of αM

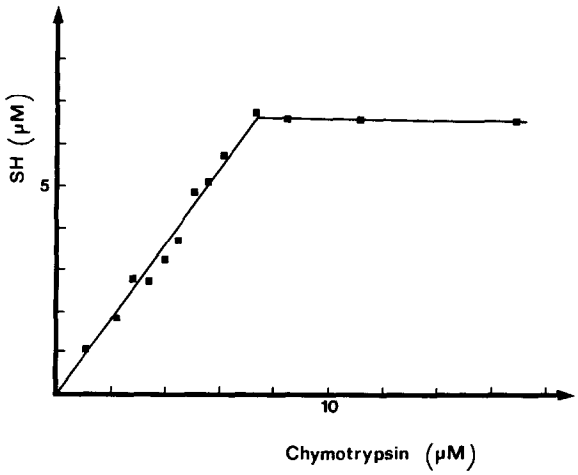

Fig.3. Titration of thiol-group appearance upon addition of chymotrypsin to α_1I_3. 70 μl of α_1I_3 (5.3×10^{-5} M) were mixed with 400–430 μl of 0.04 M Tris–HCl buffer (pH 8.0) containing 50 μl of DTNB (2.56 mM) and 0–30 μl of chymotrypsin (4.3×10^{-4} M). A_{412nm} is recorded after 1 min.

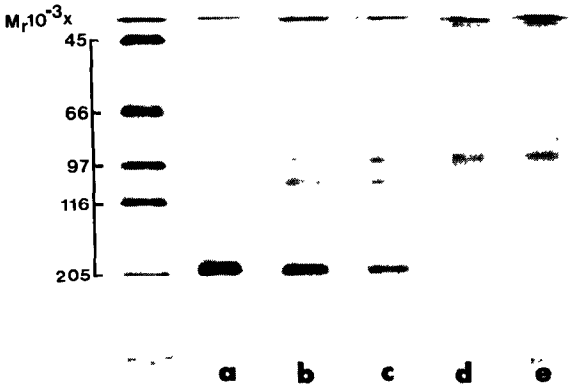

Fig.4. SDS–PAGE of reduced α_1I_3 (9 μg) incubated for 2 min at 37°C with increasing amounts of chymotrypsin. α_1I_3/chymotrypsin molar ratios were (a) 1:0; (b) 1:0.25; (c) 1:0.5; (d) 1:1; (e) 1:2.

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 αM, does not apply to α_1I_3 since the active site of α_1I_3 bound proteinases is still accessible to inhibitors of large M_r [6]. This could possibly be due to the monomeric structure of α_1I_3. A proposal has been made [19] that the non-covalent binding mode of proteinases by αM 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 α_1I_3 represents a primitive form of one or both αM 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 α_1I_3 and the quarter αM subunit may be added a functional identity since α_1I_3 proteinase complexes, like those involving αM, are rapidly eliminated from the circulation. The half-life of α_1I_3 proteinase complexes has been previously found to be about 15 min [21] whereas nascent α_1I_3 is eliminated with a half-life of 51 ± 2 h (unpublished). This is suggestive of a specific membrane receptor to proteinase modified α_1I_3, as previously found for αM. 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 α_1M and α_2M subunits as well as that of α_1I_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 α_1I_3 and the other reactive thiolester containing proteins present in plasma demonstrated, i.e., complement C_3 and C_4 [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, α_1I_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 αM species, i.e., α_2APM increases dramatically [24]. Whether or not both phenomena are related still remains to be elucidated.

Also puzzling is the fact that α_1I_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 αM ancestor in the rat is a point which has to be clarified, probably in connection with the presence of two different αM varieties in several mammal species [25].

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