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INTERACTION BETWEEN HUMAN SERUM α_1-ANTICHYMOTRYPSIN AND HUMAN LEUKOCYTE CATHEPSIN G
COMPLEX FORMATION AND PRODUCTION OF A MODIFIED INHIBITOR

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SUMMARY. The stoichiometry of the inhibition of leukocyte cathepsin G by serum α_1-antichymotrypsin was determined spectrophotometrically to be 1 : 1. Complex formation was assessed by both SDS and alkaline polyacrylamide gel electrophoreses at various molar ratios of inhibitor to enzyme ; formation of a complex with a molecular weight near 80,000 was demonstrated. Even in excess of enzyme a modified inactive form of α_1-antichymotrypsin was shown to be produced free in solution during the reaction. Moreover this modified inhibitor could not be differentiated electrophoretically and immunologically from inhibitor spontaneously dissociated from the complex.

Plasma protease inhibitors such as α_2-macroglobulin, α_1-protease inhibitor and more recently antithrombin III have been given great attention and the manners in which these inhibitors react with proteases have been investigated by several authors (1-12). With regard to the mechanism for inhibition of cathepsin G by α_1Achy, details have not been worked out. According to Travis et al. (13), the reaction results in a complex of Mr near 90,000 by SDS-PAGE. Previously they had noticed (14) that the reaction required an excess of inhibitor to inactivate all of the cathepsin G, without giving more explanations except for the possible presence of an unknown cofactor needed to activate all of the inhibitor molecules in the purified preparation.

A search for a possible mechanism of interaction between α_1Achy and cathepsin G led us to examine the products obtained for different molar ratios of these two components under various experimental conditions. This paper demonstrates that, as for antithrombin III (11), we obtain concurrently the formation of an inhibitor-enzyme complex and the appearance of a free modified inactive inhibitor. Moreover when the enzyme excess is not inactivated after formation of the complex, some degraded complex can be observed in SDS-PAGE as for antithrombin III (12).

Abbreviations: SDS, sodium dodecyl sulfate ; PMSF, phenylmethylsulfonyl fluoride ; Suc-Ala-Ala-Pro-Phe-NA,N - succinyl-L-alanyl-L-alanyl-prolyl-L-phenyl-alanyl-p-nitroanilide ; α_1Achy, α_1-antichymotrypsin ; PAGE, polyacrylamide gel electrophoresis.

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MATERIAL AND METHODS

Human serum α_1Achy was prepared by the procedure detailed previously (15). Human leukocyte cathepsin G (EC 3.4.21.20) was obtained in our laboratory from purulent sputum using the procedure described previously (16). α_1Achy was kept frozen in 0.01 M sodium phosphate buffer, 0.3 M NaCl, pH 7.5 ; the concentration of the solution (1.05 mg/ml) was measured after electroimmunodiffusion according to Weeke (17) (with dilutions of a standard serum Behring) and with the method of Lowry et al. (18). Cathepsin G, which was stored lyophilized, was dissolved just before the assays in 0.05 M sodium acetate buffer pH 5.5 containing 0.45 M NaCl. Its concentration was measured with the method of Lowry et al. (18) which gave results in good agreement with spectrophotometric measurements using an extinction coefficient ($E_{280}^{1\%}$) of 10.0 (19). The solution we used was 1.3 mg/ml.

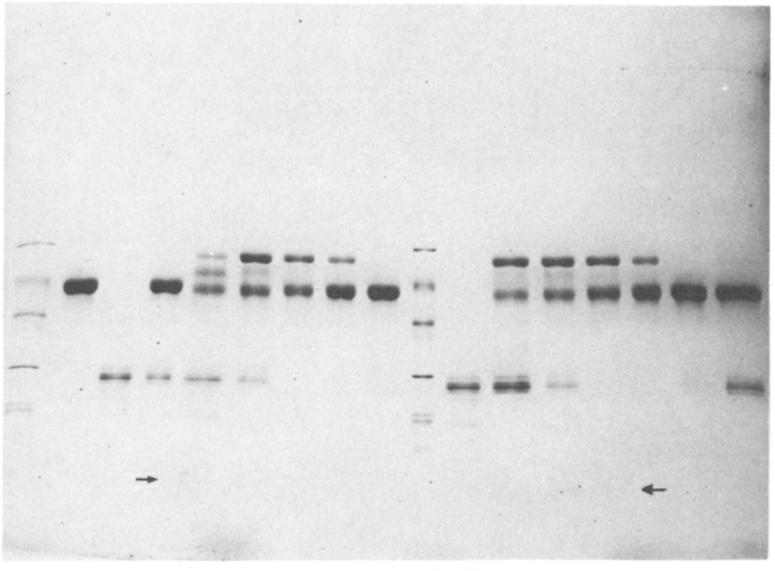
Cathepsin G activity was determined by a spectrophotometric method in which the release of p-nitroaniline from a synthetic chromogenic substrate, Suc-Ala-Ala-Pro-Phe-NA, was measured at 410 nm. The conditions were 0.1 M Hepes buffer, pH 7.5, 0.5 M NaCl, 1 % dimethylsulfoxide, 9 x 10^{-8} M enzyme and 10^{-3} M substrate (final concentrations), at 25°C. The concentration of cathepsin G was determined with the above substrate using the kinetic constants which are based on titrated enzyme (20).

Analytical electrophoreses were carried out on polyacrylamide slab gels using the technique described by Kerckaert (21). Alkaline PAGE was performed on 10 % acrylamide gels, at pH 8.3, using the gel buffer system of Davis (22). SDS PAGE was performed on a 5-30 % gel gradient using the buffer system of Laemmli (23). Before SDS-PAGE, samples were heated for 3 min in a boiling water bath, in a buffer containing 1 % SDS and 2 % mercaptoethanol. Protein precipitation and staining were performed as in our previous work (15). Mr were determined in SDS PAGE using the Pharmacia low Mr calibration kit and were estimated to be 58,000 and 27,500 for α_1Achy and leukocyte cathepsin G respectively.

RESULTS

A constant amount of cathepsin G was incubated with increasing amounts of α_1Achy for 5 min at 25°C and the remaining cathepsin G activity was measured by the spectrophotometric assay described under "Materials and Methods". The results were plotted and led to an estimation of the molar ratio between α_1Achy and cathepsin G of 1.1 : 1 at the point of full inhibition. In spite of the precautions taken during the purification procedure, a small part of the purified α_1Achy (less than 10 %) is inactive. Therefore α_1Achy and cathepsin G must form a 1 : 1 stoichiometric complex. Furthermore, we observed that α_1Achy completely inhibited cathepsin G at a molar ratio 1.1 : 1 after an incubation time as short as 1 min at 25°C. The same results were also obtained after 10 min incubation at 0°C, so these more practical conditions were used for further experiments.

Complex formation was assessed by SDS PAGE. A constant amount of α_1Achy (4 nanomoles) was incubated with varying amounts of cathepsin G. The incubation was performed for 10 min at 0°C at pH 7.5. Four different incubation mixtures were made and led to the following molar ratios of α_1Achy to cathepsin G = 0.46 : 1, 0.92 : 1, 1.85 : 1, 4.6 : 1. The remaining cathepsin G activity which was measured at the end of the incubation time for the molar ratio of 0.92 : 1 was equal to 20 % of the initial activity. That was in agreement with the previous spectrophotometric assays.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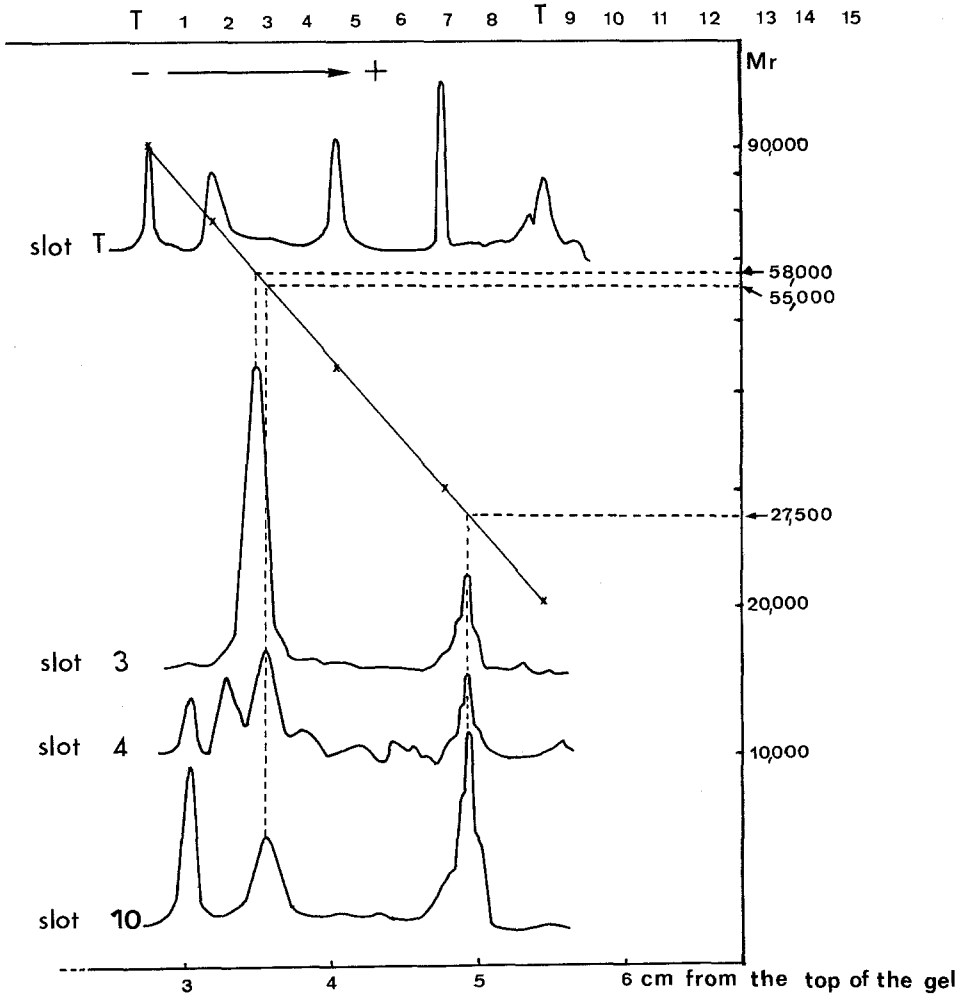
T 1 2 3 4 5 6 7 8 T 9 10 11 12 13 14 15
Mr
90,000
58,000
55,000
27,500
20,000
10,000
slot T
slot 3
slot 4
slot 10
3
4
5
6 cm from the top of the gel

An aliquot of each incubation mixture was immediately treated for SDS PAGE as described under "Materials and Methods". To the remaining part of each incubation mixture was added a hundred-fold molar excess of PMSF over cathepsin G initially present in order to stop the enzymatic action of excess cathepsin G or of cathepsin G which might have dissociated from the complex. This enzymatic action actually could occur when the samples were heated before SDS PAGE. After 15 min of contact with PMSF, an aliquot of each of these incubation mixtures was treated for SDS PAGE. The following controls were also analyzed : α_1Achy alone, cathepsin G alone and a mixture (molar ratio 1.85 : 1) of α_1Achy and cathepsin G which had been inactivated before mixing with the inhibitor by treatment with a hundred fold molar excess of PMSF for 15 min at 0°C. The SDS PAGE results are shown in Fig 1. The sample volumes analyzed were calculated to have the same initial amount of inhibitor (2 µg). We obtained the same results when mercaptoethanol was omitted during the preparation of the samples (data not shown). In slot 2 and 9, cathepsin G has not been inhibited with PMSF before the treatment for SDS PAGE, thus some autolysis occurred during this treatment and smaller components are visible. In all the incubation mixtures, we observe the presence of a component having a lower electrophoretic mobility, thus a higher Mr (estimated to be 80,000) than the native inhibitor. Since its amount in each mixture depends on the inhibitor-enzyme molar ratio, it must be a complex between α_1Achy and cathepsin G. In the mixtures with molar ratio of 0.46 : 1 a smaller amount of the 80,000 complex is detected in slot 4 than in slot 10 where PMSF was added to the mixture at the end of the incubation time. But in slot 4 another form (of Mr near 69,000) is clearly visualized. This form must be the result of degradation of the 80,000 complex, when the samples were heated, by the untreated with PMSF cathepsin G in excess. In all the incubation mixtures we can see a component which migrates in nearly the same position as the native inhibitor. When there is an excess of enzyme (Fig 1, slots 4, 5, 10 and 11) this protein band moves slightly faster than the native inhibitor ; in excess

Figure 1

SDS PAGE.

T : Mr markers : phosphorylase B (94,000), bovine albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), α-lactalbumin (14,400).

Slots 1, 8, 14 : α_1Achy. Slots 2, 9 : leukocyte cathepsin G. Slots 3, 15 : mixture of α_1Achy with previously inactivated leukocyte cathepsin G. Slots 4 to 7 : incubation mixtures without PMSF. Slots 10 to 13 : incubation mixtures with PMSF.

Molar ratios : α_1Achy : cathepsin G = Slots 4, 10 : 0.46 : 1, Slots 5, 11 : 0.92 : 1, Slots 6, 12 : 1.85 : 1, Slots 7, 13 : 4.6 : 1. (2 µg of initial inhibitor present in each slot.)

The stained gel was scanned with a densitometer Vernon PHI4. Densitometric traces are shown for slots 3, 4, 10 and T. The major band of cathepsin G was chosen as reference for the slots 3, 4 and 10 in order to compare the mobilities more precisely. The mobilities of the reference proteins (in slot T) were plotted versus the logarithm of their molecular weight. Then the comparison of the molecular weight of the two forms of α_1Achy was made with cathepsin G (Mr : 27,500) as reference.

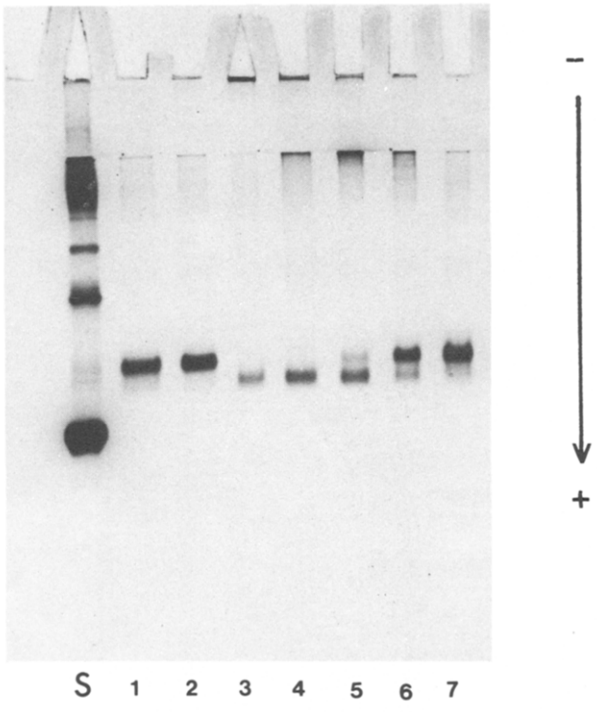

Figure 2
Alkaline PAGE
S : normal serum. Slots 1, 7 : α_1Achy. Slot 2 : mixture of α_1Achy and previously inactivated cathepsin G. Slots 3 to 6 : incubation mixtures with PMSF. Molar ratios α_1Achy : cathepsin G = slot 3 = 0.46 : 1, slot 4 = 0.92 : 1, slot 5 = 1.85 : 1, slot 6 = 4.6 : 1. (3 µg of initial inhibitor present in each slot.)

of inhibitor (Fig 1, slots 6, 7, 12 and 13) this band is broader and consists of a mixture of the native inhibitor and the other faster migrating component. Densitometric traces of slots 3, 4 and 10 are shown as the trace of slot T which allows us to estimate the Mr difference between the slightly faster band and the native inhibitor : about 3,000. In the mixtures with active cathepsin G we are able to observe a diffuse band of Mr near 6,000, slightly stained with Coomassie Blue (marked with an arrow, Fig 1).

The incubation mixtures treated with PMSF were studied in alkaline PAGE (Fig 2). The mixture of α_1Achy with inactivated cathepsin G gives the same pattern as α_1Achy alone (Fig 2, slots 1 and 2). At pH 8.3 cathepsin G (pI > 10) is not able to migrate towards the anode. The volumes of the analyzed samples were calculated to have the same initial amount of inhibitor in each slot (3 µg). In the first two mixtures (molar ratios 0.46 : 1, 0.92 : 1) (Fig 2, slots 3 and 4) the band corresponding to the native inhibitor has disappeared. We only can see a more lightly stained band which moves faster than the native inhibitor. Its pI, determined by isoelectric focusing, is equal to 3.75 instead of 3.9 for native inhibitor. In crossed immunoelectrophoresis these samples give a precipitation curve with specific antiserum

against α_1Achy with α_1 mobility at pH 8.6 (data not shown). This faster band corresponds to a modified form of α_1Achy. In the last two mixtures (slots 5 and 6), when there is an excess of inhibitor, native inhibitor still exists in addition to the faster band. Contrary to the faster band, this native inhibitor has kept the ability to inactivate cathepsin G or bovine chymotrypsin when we use the method for characterization of the activity of protease inhibitors previously described (15). In this electrophoretic system the complex cannot be easily shown because it tends to aggregate : the mixture solutions are more turbid than the initial inhibitor and enzyme solutions. Thus this aggregated complex does not easily enter the gel.

In order to know how the reaction was going on, a mixture with molar ratio of 1.85 : 1 was left in the buffer used for the complex formation (pH 7.5) without PMSF at 4°C for 7 days. As control native inhibitor was kept in the same conditions. After 24 h some complex persisted, after 7 days the complex previously shown by SDS PAGE (Fig 1 slot 6) has completely disappeared (Fig 3A, slot 1) ; the following components are seen : one band migrating slightly faster than the native inhibitor (of Mr near 55,000) and the diffuse band of Mr near 6,000 that we have seen before. When the mixture was studied in alkaline PAGE (Fig 3B, slot 1) and compared to the same mixture left for 10 mn at 0°C (Fig 2, slot 5) only the faster component was visualized. This component reacted with antiserum against α_1Achy.

DISCUSSION. The spectrophotometric assays bring to the fore the fact that equimolar amounts of α_1Achy and cathepsin G are required for full inhibition of the enzyme. The complex formed has a Mr (80,000) in SDS PAGE smaller than the one expected (58,000 + 27,500 = 85,500) ; however its Mr confirms a 1 : 1 stoichiometric reaction. The diffuse band of Mr near 6,000 corresponds probably to the missing peptide. Besides these two components, in the presence of excess enzyme, while all of the inhibitor should be complexed, an unexpected band is observed after SDS PAGE, having a slightly faster mobility than the native inhibitor. Considering the results obtained in alkaline PAGE, without dissociation agent, this form does not represent the result of a partial complex dissociation due to the presence of SDS in the gel and in the samples. Actually, in these incubation mixtures no native inhibitor is found using the alkaline PAGE, only a new faster moving component is detected ; it reacts with antiserum against α_1Achy but has lost the ability to inhibit cathepsin G or bovine chymotrypsin. This modified α_1Achy is probably a product of cathepsin G action because none of it is formed when α_1Achy is incubated with cathepsin G which had been previously inactivated with PMSF.

In the last experiment, we demonstrated that a dissociation or a degradation of the complex occurred spontaneously in 7 days ; it leads to a

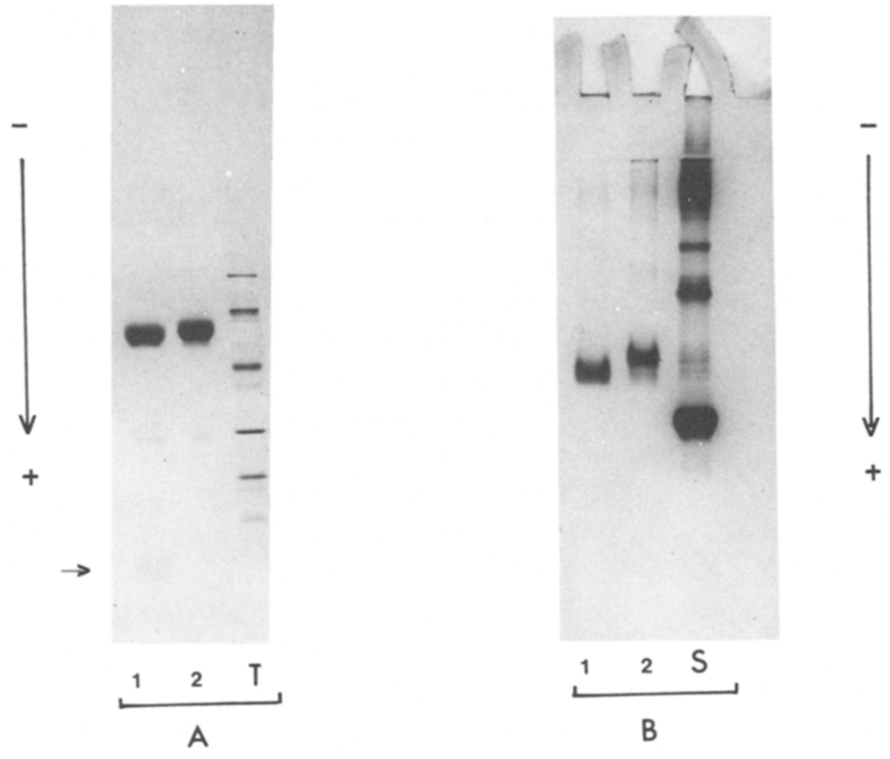

Figure 3
PAGE patterns of a mixture of α_1Achy and leukocyte cathepsin G molar ratio 1.85 : 1 kept for 7 days at 4°C at pH 7.5 (slots 1), and of α_1Achy alone (slots 2).
A - SDS PAGE. T = Mr markers (the same as in Fig. 1). (the diffuse band with a molecular weight of about 6,000 is marked with an arrow).
B - Alkaline PAGE. S = normal serum.

modified form of α_1Achy, which cannot be electrophoretically and immunologically differentiated from the modified inhibitor detected after 10 min incubation at 0°C.

All these data suggest that as for the interaction between antithrombin III and thrombin (24), limited proteolysis of the inhibitor by the enzyme may be involved in the mechanism by which α_1Achy inhibits leukocyte cathepsin G. Such a mechanism has been much debated in the interaction between α_1-protease inhibitor and proteases (5-8). Tentative hypothesis given by Fish and Björk (24) for the mode of action of antithrombin III in the inhibition of thrombin would seem to be suitable, in the broad lines, in this case. The ratios between the two possible ways these authors have suggested for the modified inhibitor present in the initial complex are probably depending on the association and dissociation rate constants of the reactions.

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