Biochimica et Biophysica Acta, 613 (1980) 469—475

BBA 69010

STUDIES ON THE COMPLEX BETWEEN HUMAN α_2-MACROGLOBULIN AND *CROTALUS ADAMANTEUS* PROTEINASE II

RELEASE OF ACTIVE PROTEINASE FROM THE COMPLEX *

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(Received November 5th, 1979)

Key words: α_2-Macroglobulin; Proteinase II; Protein complex

Summary

Proteinase II from *Crotalus adamanteus* venom formed a complex with human α_2-macroglobulin in which approx. 1.7 mol of enzyme were bound per mol inhibitor. The complex did not enzymatically inactivate human α_1 proteinase inhibitor. However, active proteinase II was released from the complex in the presence of a high molecular weight proteinase fraction from *C. adamanteus* venom. The α_2-macroglobulin-proteinase II complex was also unstable during incubation in serum, and the enzyme released from the complex caused inactivation of serum proteinase inhibitors.

The results indicate conditions under which venom proteinases can be dissociated from their complexes with α_2-macroglobulin and thus remain functional in the presence of molar excesses of inhibitor.

Introduction

A previously undescribed property of certain snake venom metallo-proteinases, namely, the ability to enzymatically inactivate all trypsin and chymotrypsin inhibitory activity of human serum has been reported [1]. This differs from the usual interaction of proteinases with inhibitors, in which a stable complex is formed [2]. Two enzymes from *Crotalus adamanteus* venom responsible for the inactivation of human plasma α_1 proteinase inhibitor were purified to homogeneity and designated proteinases I and II [3]. Proteinase II

* Dedicated to Professor Michael Laskowski, Sr., on his 75th birthday.
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inactivated α_1 proteinase inhibitor by limited proteolysis of an X-Met bond [4], which differed from the bond in α_1 proteinase inhibitor cleaved by other proteinases [5].

The original report [1] noted that more venom was needed to inactivate inhibitors in serum than was employed to inactivate α_1 proteinase inhibitor. When less venom was incubated with serum, a delay occurred before the inactivation of serum inhibitors began. It was postulated that this effect might be due to formation of a transient complex between venom proteinases and serum α_2-macroglobulin. However, no direct evidence for this was presented [1].

Human α_2-macroglobulin is a plasma proteinase inhibitor which forms complexes with virtually all proteolytic enzymes [6,7]. Typically, α_2-macroglobulin-enzyme complexes are stable and retain the ability to digest small peptide substrates, but are inactive toward large proteins [6—8]. α_2-Macroglobulin is known to form complexes with proteinases involved in coagulation [9,10], fibrinolysis [11] and kinin formation [12], but its role in the regulation of these processes is not well defined [13]. Although proteinases affecting the above processes are widely distributed among snake venoms [14,15], the effect of α_2-macroglobulin on the proteolytic activity of venom has not been extensively studied. Several reports on the interaction between α_2-macroglobulin and purified venom enzymes which clot fibrinogen ('thrombin-like esterases') have appeared [16,17]. However, the effect of α_2-macroglobulin on other types of purified venom proteinases has apparently not been investigated, and no studies have been made of the behavior of the α_2-macroglobulin-venom proteinase complexes in the presence of other venom enzymes.

The present report shows that *C. adamanteus* proteinase II reacts with α_2-macroglobulin to form a complex in which proteinase II has lost the ability to digest α_1 proteinase inhibitor. The release of active proteinase II from the complex in the presence either of serum or of a high molecular weight *C. adamanteus* proteinase fraction is described.

Materials and Methods

Human serum and plasma were obtained through Dr. Elias Cohen, Plasmapheresis Department, Roswell Park Memorial Institute. *C. adamanteus* venom was purchased from Miami Serpentarium. α_2-Macroglobulin and α_1 proteinase inhibitor were prepared in this laboratory [18]. Assays for enzymes and for inhibitor inactivation were performed as described previously [1]. α_2-Macroglobulin activity [19] was measured using benzoyl-L-arginine ethyl ester. *C. adamanteus* proteinase II was prepared as described [3]. Peak I proteinase from *C. adamanteus* was prepared by gel filtration of the crude venom on Bio-Gel P-150 [3]. The high molecular weight proteinases in the first peak were pooled, lyophilized and used without further purification.

Results

Stoichiometry of the α_2-macroglobulin-proteinase II complex

Increasing amounts of proteinase II were incubated for 1 min with α_2-macro-

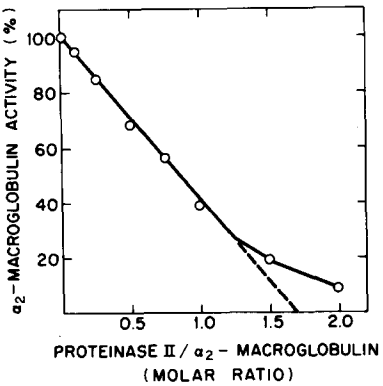

Fig. 1. Stoichiometry of complex formation between proteinase II and α_2-macroglobulin. Increasing amounts of proteinase II (0.9—18.5 μg) were added to 280 μg of α_2-macroglobulin (both in 0.05 M Tris-HCl, pH 8) in a total volume of 100 μl of the same buffer. After 1 min at 23° C, aliquots of each incubation mixture were assayed for residual α_2-macroglobulin activity [19]. The molar ratios were calculated based on molecular weights of 725 000 for α_2-macroglobulin [7] and 24 000 for proteinase II [3].

globulin as shown in Fig. 1. The decrease in α_2-macroglobulin activity indicates formation of an enzyme-inhibitor complex. Extrapolation of the linear portion of the inhibition curve shows that 1 mol of α_2-macroglobulin binds approx. 1.7 mol of proteinase II. Proteinase II inactivated by exposure to 0.01 M EDTA, pH 8.0 [3], was reacted with α_2-macroglobulin at a ratio of 2.0 mol enzyme per mol α_2-macroglobulin. An 8% decrease in α_2-macroglobulin activity was observed, indicating a slight effect due to nonspecific binding. Therefore, complex formation is due predominantly to binding of the active form of the enzyme.

The complex formed with 0.5 mol of proteinase II per mol α_2-macroglobulin was tested for α_2-macroglobulin activity over a period of 20 h. Immediately after the addition of proteinase II, the α_2-macroglobulin activity decreased to 68% of its starting value. However, no further changes in α_2 macroglobulin activity were noted. This result is expected if proteinase II is forming a stable complex with α_2-macroglobulin rather than enzymatically inactivating the inhibitor, as was noted with α_1 proteinase inhibitor [1,3]. The α_2-macroglobulin-proteinase II complex showed no inactivation of α_1-proteinase inhibitor, indicating that the active site of the proteinase for this large protein substrate has been masked by α_2-macroglobulin.

Effect of high molecular weight C. adamanteus proteinase fraction on the α_2-macroglobulin-proteinase II complex

The α_2-macroglobulin-proteinase II complex formed with α_2-macroglobulin in slight excess showed no detectable release of proteinase II when incubated with α_1 proteinase inhibitor, and full α_1 proteinase inhibitor activity was retained (Fig. 2). The high molecular weight peak I proteinase fraction from *C. adamanteus* was then incubated with α_1 proteinase inhibitor. No decrease in inhibitory activity was noted during 3 h of incubation. Thus, the α_2-macroglobulin-proteinase II complex or the peak I proteinase fraction do not by

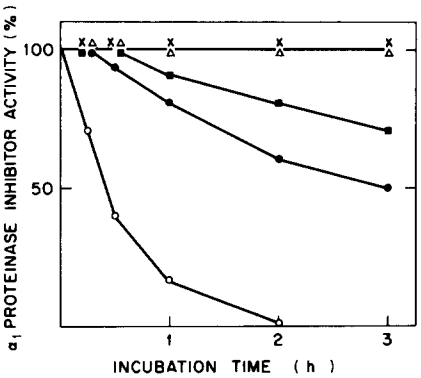

Fig. 2. Effect of *C. adamanteus* peak I proteinase fraction on the α_2-macroglobulin-proteinase II complex. α_2-Macroglobulin (117 μg) was incubated with proteinase II (5.7 μg) at pH 8.0, 23°C. After 3 min α_1 proteinase inhibitor (122 μg) was added, followed by peak I proteinase fraction. The final volume was 800 μl. Aliquots were withdrawn, diluted with 0.05 M Tris, 0.01 M EDTA, pH 8.0, and the α_1 proteinase inhibitor assayed for residual trypsin inhibitory activity [1]. X———X, α_2-macroglobulin-proteinase II complex; △———△, peak I proteinase fraction; ■———■, complex plus 3 μg peak I proteinase fraction; ●———●, complex plus 6 μg peak I proteinase fraction; ○———○, proteinase II. All values are normalized to the activity of control samples containing α_1 proteinase inhibitor and trypsin only.

themselves inactivate α_1 proteinase inhibitor under the experimental conditions.

However, when the peak I proteinase fraction was added to the α_2-macroglobulin-proteinase II complex in the presence of α_1 proteinase inhibitor, proteinase II was released from the complex, and inactivation of α_1 proteinase inhibitor proceeded after an initial delay of 15—30 min (Fig. 2). Peak I proteinase fraction released sufficient proteinase II from the complex to give 30—50% inactivation of α_1 proteinase inhibitor after 3 h. A control containing an amount of uncomplexed proteinase II equivalent to that present in the complex gave 100% α_1 proteinase inhibitor inactivation within 2 h. This indicates either that an incomplete release of proteinase II from the complex occurs under the conditions used, or that the proteinase II which was released does not catalyze α_1 proteinase inhibitor inactivation as efficiently as the intact enzyme.

Transient nature of the α_2-macroglobulin-proteinase II complex in serum

The addition of increasing amounts of proteinase II to serum (Table I) results in a corresponding decrease in the serum α_2-macroglobulin level measured after 1 min incubation. For a given amount of proteinase II, the level of α_2-macroglobulin after 6 h incubation is approx. the same as that measured at 1 min. No enzymatic inactivation of α_2-macroglobulin has occurred, and an excess of α_2-macroglobulin was present during the entire 6 h incubation period for all levels of proteinase II tested.

However, the chymotrypsin inhibitory activity levels shown in Table I indicate that the α_2-macroglobulin-proteinase II complex is unstable in serum. No decrease in serum chymotrypsin inhibitor activity is noted for at least 15 min. Then, active proteinase II is released from the complex and serum inhibitor

TABLE I

EFFECT OF INCUBATION IN SERUM ON THE α_2-MACROGLOBULIN-PROTEINASE II COMPLEX

Increasing amounts of proteinase II were added to 400 μl of serum at 23°C in 0.05 M Tris-HCl-0.002 M $CaCl_2$, pH 8.0, to a final volume of 650 μl. Aliquots were withdrawn and measured for residual α_2-macroglobulin activity [19] after 1 min and 6 h of incubation. Release of active proteinase II during the incubation period was monitored by assaying aliquots diluted with Tris-EDTA for residual serum chymotrypsin inhibitory activity [1] at the times indicated.

Amount of proteinase II added to serum (μg)	Serum α_2-macroglobulin remaining (%)		Serum chymotrypsin inhibitory activity remaining at various times after proteinase II addition (%)			
min:	1	360	1	15	30	360
0	100	100	100	—	—	100
0.2	73	70	100	100	100	100
0.4	60	61	100	100	100	95
0.6	52	53	100	100	69	13
0.8	45	44	100	100	56	0

inactivation proceeds. With the highest level of proteinase II added (0.8 μg), a total loss of serum inhibitory activity was noted after 6 h. With 0.4 and 0.6 μg of proteinase II, total loss was observed after 20 h (not shown). In contrast, when 0.2 μg of proteinase II were incubated with serum, no loss of inhibitory activity was noted after 20 h. Apparently, a slight amount of proteinase II is being more tightly bound to α_2-macroglobulin, and is not released under the experimental conditions.

The observed loss of serum chymotrypsin inhibitory activity is predominantly due to limited proteolysis of α_1 proteinase inhibitor [3], the major inhibitor in serum. If only α_1 proteinase inhibitor were present or only enzymatic inactivation were involved, an immediate and rapid decrease in chymotrypsin inhibitory activity would be expected with the amounts of venom used [1]. The results are consistent with the interpretation that a transient complex is formed between proteinase II and α_2-macroglobulin in serum.

Discussion

Formation of a stoichiometric enzyme-inhibitor complex with α_2-macroglobulin is in contrast to the action of proteinase II on other plasma proteinase inhibitors [1,3] in which enzymatic digestion by the proteinase results in a gradual loss of all inhibitory activity. With α_2-macroglobulin the decrease in inhibitory activity corresponds to that expected for complex formation with proteinase II, and no further enzymatic digestion of α_2-macroglobulin occurs. The 1.7 : 1 stoichiometry for proteinase II and α_2-macroglobulin (Fig. 1) is consistent with the reports that α_2-macroglobulin binds 1.7 to 2.1 mol of trypsin [20] or 2 mol of chymotrypsin [21], but differs from earlier reports indicating a 1 : 1 binding stoichiometry [7,22].

In the absence of external factors, proteinase II remains tightly bound to

α_2-macroglobulin, and the complex is unable to digest α_1 proteinase inhibitor. With respect to these properties the α_2-macroglobulin-proteinase II complex resembles other α_2-macroglobulin-enzyme complexes [6,7]. The release of proteinase II from the α_2-macroglobulin complex in serum or in the presence of peak I proteinase fraction could have functional significance, since this property would allow retention of in vivo proteolytic activity despite a molar excess of circulating α_2-macroglobulin. In addition, the α_2-macroglobulin complex could conceivably be transported from the immediate snakebite area and active proteinase II subsequently released at remote sites. Although the experiments in the present study were done at 23°C, other results (Kress, L.F., unpublished data) show that α_2-macroglobulin and serum react with venom proteinases at 37°C under conditions approximating those present in vivo.

Proteinase II appears to have a higher affinity for α_2-macroglobulin than for other plasma proteinase inhibitors (Table I). However, once proteinase II has been released from the α_2-macroglobulin complex, there is no tendency to reassociate with α_2-macroglobulin. This may indicate that proteinase II is released as an active enzyme attached to an α_2-macroglobulin fragment as has been observed with the α_2-macroglobulin-trypsin complex [23]. Presence of the fragment would prevent reformation of the complex, and could also account for the slower rate of α_1 proteinase inhibitor inactivation (Fig. 2).

The peak I proteinase fraction could either be displacing proteinase II from the complex or digesting the α_2-macroglobulin, causing release of active proteinase II. Since the peak I proteinase fraction is only partially purified, the data presented do not exclude the possibility that several proteinases may be acting on the α_2-macroglobulin complex. Alternatively, nonproteolytic contaminants in peak I could be responsible, but this seems unlikely. Studies are in progress to further purify the peak I proteinases and determine their mechanism of action on the α_2-macroglobulin-proteinase II complex.

Previously, it was reported that α_2-macroglobulin irreversibly bound all endopeptidases studied [6,24], although nonbinding of kallikreins has been observed [25]. Furthermore, no bound proteinase could be displaced from the α_2-macroglobulin complex by the addition of the same or another proteinase [24]. The release of active proteinase II from the α_2-macroglobulin complex therefore, is an unexpected observation. It is possible that the binding characteristics of proteinase II resemble those of carboxypeptidase A which is reversibly bound to α_2-macroglobulin [6]. However, proteinase II appears to be more specific in its α_2-macroglobulin binding than does carboxypeptidase A. Unlike carboxypeptidase A, the binding of proteinase II to α_2-macroglobulin is not reversed by the presence of serum albumin (Table I), and α_2-macroglobulin does not bind significant amounts of inactive proteinase II.

Acknowledgements

The authors wish to thank Dr. M. Laskowski, Sr., Head of the Laboratory of Enzymology, for his continuing interest and support of this project. The technical assistance of E.A. Paroski and J. Catanese is also gratefully acknowledged. The research was supported by grants HL 22996 (L.F.K.) and HL 15892 (M. Laskowski, Sr.) from the National Institutes of Health.

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