ON THE STRUCTURE OF THE STABLE COMPLEX BETWEEN PLASMIN AND α_2-ANTIPLASMIN

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1. Introduction

Many of the protease inhibitors in plasma form extremely stable complexes with proteolytic enzymes, which cannot be dissociated by strong denaturating agents [1–4]. Many of these complexes are, however, dissociated by nucleophilic agents; a covalent bond may be involved in their stabilization [1,2]. This has also been suggested for inhibitors such as soybean trypsin inhibitor and aprotinin in their complexes with proteases [5,6]. The reaction between plasmin and α_2-antiplasmin was investigated by a number of kinetic [7–9] and structural studies [10]. The kinetic data have revealed a two-step reaction: (i) a very fast reversible second-order reaction; followed by (ii) a slower irreversible transition [7–9]. The structural data have indicated that the reactive site in α_2-antiplasmin constitutes a specific leucyl–methionyl residue in the carboxy-terminal portion of the molecule [10]. α_2-Antiplasmin and its interactions are reviewed in [11]. This study was done to investigate whether the reactive site in α_2-antiplasmin is cleaved as a result of complex formation with plasmin. Part of this work has been presented in [12].

2. Materials and methods

2.1. *Plasminogen*

Glu–plasminogen was purified by affinity chromatography on lysine–Sepharose [13] followed by DEAE–Sephadex A-50 chromatography [14]. It was characterized by SDS–polyacrylamide gel electrophoresis and NH_2-terminal amino acid determination.

2.2. *α_2-Antiplasmin*

α_2-Antiplasmin was purified by affinity chromatography on LBS I–Sepharose followed by gel-filtration on Ultrogel AcA 44, as in [11,15]. LBS I is a fragment from plasminogen constituting the 3 NH_2-terminal triple-loop structures obtained after digestion with elastase [16]. The material was characterized by SDS–polyacrylamide gel electrophoresis and NH_2-terminal amino acid analysis and found to fulfill the specifications in [15].

2.3. *Complexes between Glu–plasmin and protease inhibitors*

To obtain Glu–plasmin complexes with α_2-antiplasmin or soybean trypsin inhibitor, a mixture of Glu–plasminogen (final conc. ~10 mg/ml) and the inhibitor in ~50% molar excess were dissolved in 0.1 M sodium phosphate buffer (pH 7.3). Activation was performed by the addition of urokinase (final conc. 2500 IU/ml) and incubation at room temperature for 2 h. The material was subsequently dialyzed extensively against distilled water at +5°C and lyophilized.

2.4. *Reagents*

Soybean trypsin inhibitor was purchased from Sigma (St Louis MO). Urokinase was a gift from Choay (Paris, courtesy of Dr F. Toulemonde). $Na^{125}I$ was from Kebo AB (Stockholm) and ^3H-labelled phenylisothiocyanate from The Radiochemical Centre (Amersham). All other reagents were of analytical grade.

2.5. *SDS–polyacrylamide gel electrophoresis*

This was performed essentially as in [17] using 7% polyacrylamide gels on reduced as well as non-reduced samples. After staining, the gels containing ^{125}I-labelled

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material were cut into slices representing the different interesting protein bands and the radioactivity was counted in a Multigamma counter (LKB, Stockholm).

2.6. *NH_2-terminal amino acid analysis*

NH_2-Terminal amino acids were determined by manual Edman degradation essentially as in [18]. The phenylthiohydantoin amino acid derivatives were identified by thin-layer chromatography on silica gel plates, as in [19]. Radioactive derivatives were quantitated by scraping off the silica containing the different spots, adding 5 ml scintillation liquid (Instagel, Packard Instruments) and counting in a liquid counter (Intertechnique, SL4000). Quantitation of amino acid phenylthiohydantoin derivatives were performed by amino acid analysis (LKB, Biochrom 4400) after hydrolysis for 4 h at 150°C in 6 M HCl containing 0.1% stannous chloride [20].

3. Results

3.1. *NH_2-terminal amino acids in soybean trypsin inhibitor or α_2-antiplasmin in complex with plasmin*

As a first step to find out the nature of the stabilizing bond in different plasmin–inhibitor complexes, these were subjected to NH_2-terminal amino acid determination. About equal amounts of glutamic acid, valine and aspartic acid in ~1 mol/mol soybean trypsin inhibitor–plasmin complex were found (table 1). This represents the NH_2-terminal amino acid in the parent molecules. However, in the plasmin–α_2-antiplasmin complex, besides the expected NH_2-terminal amino acids (glutamic acid, valine and aspargine) methionine was also found at ~1 mol/mol complex.

Table 1

Amino acid	a	b	c	d
Aspartic acid	–	1.5	2.2	1.0
Glutamic acid	0.9	0.7	0.7	–
Valine	–	1.0	1.0	–
Methionine	0.1	0.1	1.0	–
Isoleucine	–	0.1	0.1	–

NH_2-terminal amino acids in: plasminogen (a); the complex between plasmin and soybean trypsin inhibitor, obtained by activation of plasminogen in ~50% molar excess of the inhibitor (b); plasmin–α_2-antiplasmin complex obtained by incubation (16 h) of the plasmin–soybean trypsin inhibitor complex (50% excess inhibitor) with an equimolar amount of α_2-antiplasmin (c); soybean trypsin inhibitor obtained after dissociation of the soybean trypsin inhibitor–plasmin complex by α_2-antiplasmin (d). Figures are expreseed in mol/mol protein (a,d) or mol/mol complex (b,c)

3.2. *Dissociation of soybean trypsin inhibitor–plasmin complex in the presence of α_2-antiplasmin*

If soybean trypsin inhibitor–plasmin complex is mixed with α_2-antiplasmin in molar excess the plasmin is completely transferred into a complex with α_2-antiplasmin. The NH_2-terminal amino acids in such a complex are demonstrated in table 1. By gel-filtration on Ultrogel AcA 44 in 0.1 M NH_4HCO_3 the plasmin–α_2-antiplasmin complex can be separated from the released soybean trypsin inhibitor. Only NH_2-terminal aspartic acid is found in the latter inhibitor-moiety, which is identical to the native soybean trypsin inhibitor preparation. Furthermore, analysis of the released soybean trypsin inhibitor by SDS–polyacrylamide gel electrophoresis (reduced and non-reduced samples) demonstrated a single chain molecule with a mobility identical to that of the native molecule. Analysis of its inhibitory activity against plasmin revealed that ~90% activity was retained.

To exclude the possibility of secondary proteolytic cleavage by plasmin as a cause of peptide bond cleavage in α_2-antiplasmin during its reaction with plasmin, the following experiments were undertaken. ^{125}I-Labelled Glu–plasmin in complex with soybean trypsin inhibitor was dissolved in 4.5% (v/v) dimethylallylamine buffer (adjusted with trifluoroacetic acid to pH 8.0) to 10 mg/ml final conc. and mixed with an equal volume of α_2-antiplasmin solution (9 mg/ml in the same buffer). At different times, over 0–4 h, samples were taken for SDS–polyacrylamide gel electrophoresis and for NH_2-terminal amino acid determination. After staining the gels, the bands comprising plasmin and plasmin–α_2-antiplasmin complex were cut out and counted for radioactivity. From the results, shown in fig.1, the dissociation of the soybean trypsin inhibitor–plasmin complex was found to obey first-order kinetics with a half-life of ~60 min, which equals a rate constant of 2×10^{-4} s^{-1}. Within experimental error, the appearance of methionine as new NH_2-terminal amino acid is concomitant with the formation of the plasmin–α_2-antiplasmin complex (fig.1).

3.3. *Pulse labelling of plasmin–α_2-antiplasmin with [^{3}H]phenylisothiocyanate*

In an attempt to show that NH_2-terminal methionine is not produced during the coupling step of the

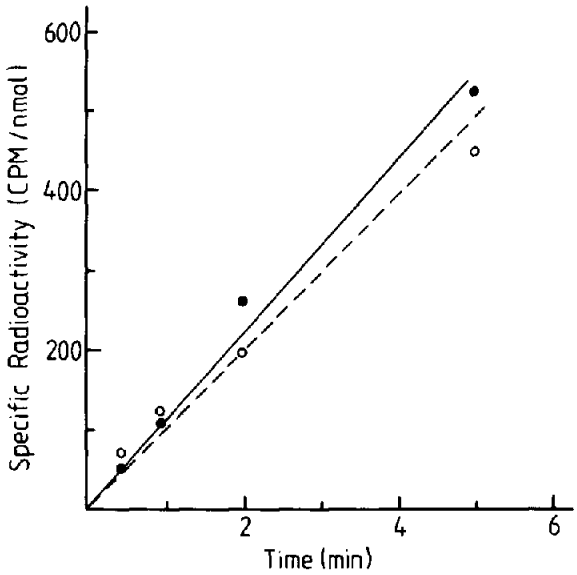

Fig.1. Dissociation of the plasmin–soybean trypsin inhibitor complex in the presence of α_2-antiplasmin as demonstrated by the formation of plasmin–α_2-antiplasmin complex (o——o) and the appearance of new NH_2-terminal methionine (•——•). For details of the experiments see text. The figures are given as ratios between the plasmin–α_2-antiplasmin complex/the initial plasmin–soybean trypsin inhibitor complex and methionine/valine, respectively.

NH_2-terminal amino acid analysis, but exists in the native complex, a pulse-labelling experiment with [^{3}H]phenylisothiocyanate was done. For this purpose plasmin–α_2-antiplasmin complex was dissolved in 0.01 M sodium phosphate buffer (pH 7.8) to 20 mg/ml final conc. and divided into 250 μl aliquots. A solution (0.5 ml) of pyridine and dimethylallylamine (15/1, v/v) adjusted with trifluoroacetic acid to pH 9.2, containing 0.1 μl [^{3}H]phenylisothiocyanate/ml was added at 45°C. After a certain reaction time, from 0.5–5 min, 100 μl non-labelled phenylisothiocyanate was added and incubation was carried out for another 60 min at 45°C in a nitrogen atmosphere. Subsequently, a normal NH_2-terminal amino acid analysis was performed. The specific radioactivity of the different NH_2-terminal amino acids derivatives were plotted against the reaction time (fig.2). The rate of the labelling of NH_2-terminal methionine, within experimental errors, equals that of the other NH_2-terminal amino acids (Asn, Asp, Glu, Val) in the complex, indicating its existence already in the native complex. For simplicity only the results with methionine and valine are demonstrated in fig.2.

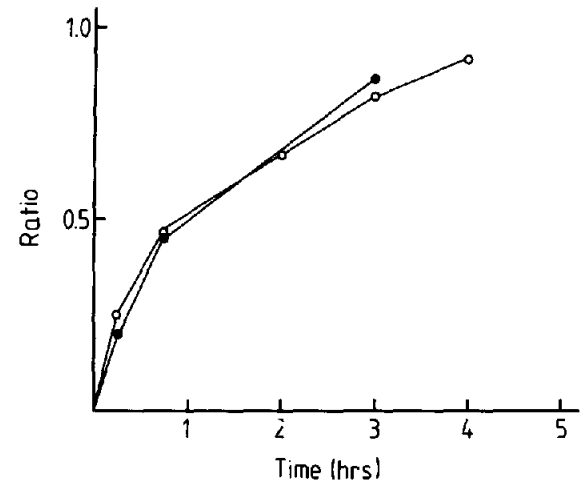

Fig.2. Rate of [^{3}H]phenylisothiocyanate labelling of methionine (▪——▪) as compared to valine (o– – –o) in the plasmin–α_2-antiplasmin complex. For details of the experiment see text.

4. Discussion

Structural studies on the plasmin–α_2-antiplasmin complex have indicated that the reactive site in α_2-antiplasmin constitutes a specific peptide bond in the carboxy-terminal portion of the molecule [10–12]. A peptide of ~8000 M_r and NH_2-terminal methionine, originating from the carboxy-terminal end of α_2-antiplasmin could be isolated by gel-filtration of the complex in the presence of SDS or after treatment with diluted NH_4OH [10]. The peptide could, however, not be isolated by gel-filtration in 10% acetic acid or in buffers containing urea or guanidinium–HCl. Therefore, we undertook this work to investigate whether the reactive-site peptide bond in α_2-antiplasmin becomes cleaved as a result of complex formation with plasmin. From indirect evidence it had been suggested that an ester-bond may be of importance in the stabilization of many protease–inhibitor complexes, since they are cleaved by agents such as hydroxylamine, benzamidine, weak alkali or methoxyamine [1,2,10,21]. Of course, a tetrahedral intermediate could not be excluded from these results.

This study demonstrates a difference in the binding of plasmin to α_2-antiplasmin as compared to soybean trypsin inhibitor, the former being much more stable. Dissociation of the plasmin–soybean trypsin inhibitor complex by α_2-antiplasmin releases an intact form of soybean trypsin inhibitor. Furthermore, our data show that formation of the stable complex between

plasmin and α_2-antiplasmin is accompanied by cleavage of the reactive-site peptide bond, since the appearance of NH_2-terminal methionine parallels the complex formation (fig.1).

Crystallographic data have suggested that complexes between trypsin and inhibitors such as soybean trypsin inhibitor or aprotinine are stabilized by the formation of a tetrahedral intermediate [5,6]. These data were, however, not supported by ^{13}C NMR studies [22], which rather indicated that non-covalent bonds stabilize these inhibitor–protease complexes. Our data exclude a tetrahedral intermediate as being important in the plasmin–α_2-antiplasmin interaction. An ester-bond between the hydroxyl-group in the active site of plasmin and the carboxyl-group of the peptide bond in the reactive site is much more plausible, in agreement with earlier speculations [1,2]. However, the explanation for the stability of this ester-bonded intermediate is not known, but several mechanisms are possible. One explanation may be that a very tight complex is formed which does not leave any space for the water molecule necessary to hydrolyze the ester-bond. However, it has been reported that the complex between, e.g., thrombin and antithrombin III can be dissociated by benzamidine or hydroxylamine [1,2] for example. These substances do not interfere with the plasmin–α_2-antiplasmin complex at physiological pH under non-denaturating condition. Nevertheless, in the presence of SDS, hydroxylamine seems to cleave the plasmin–α_2-antiplasmin complex (Wiman and Collen, unpublished). Another possible explanation of the stability of the ester-bond is that cleavage of the reactive site peptide bond results in conformational changes, thus interfering with the electron-relay system within the catalytic center of the enzyme. We have shown that conformational changes are likely to occur as a result of complex formation between and α_2-antiplasmin as studied by immunochemical [23,24] or spectropolarimetric investigations [25]. Work to isolate a fragment from the complex containing the bond between plasmin and α_2-antiplasmin is in progress.

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