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Determination of intermediates, products and cleavage site in the reaction between plasminogen activator inhibitor type-2 and urokinases

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Several specific inhibitors for plasminogen activators have been isolated from various organs and cell lines, those from human placenta and the human monocyte-like cell line U-937 being virtually identical. The reaction between this type of inhibitor, designated as type-2, and high-M_r and low-M_r urokinase-type plasminogen activators was followed by reversed-phase high-performance liquid chromatography and gel electrophoresis. The components, their stable complexes and their dissociation and cleavage products could be clearly identified in both systems. The amino acid sequence of the inhibitor at the cleavage site was determined to be -Met-Thr-Gly-Arg↓Thr-Gly-His-Gly-. A 35-residue carboxy-terminal fragment was found to be released.

Plasminogen activator inhibitor; Plasminogen activator; Serpin; Urokinase; HPLC; Amino acid sequence

1. INTRODUCTION

Plasminogen activators belong to the family of serine proteinases and are responsible for the conversion of the proenzyme plasminogen to its active form, plasmin. Two physiologically important activators are known: urokinase-type (u-PA) and tissue-type activator (cf. [1]). Specific inhibitors (PAI), which lack a direct effect on plasmin, regulate the activity of the activators. Recently, such inhibitors have been isolated from several organ types and cell line cultures (cf. [2]). The inhibitors purified from human placenta [3,4] and from the human monocyte-like histiocytic lymphoma cell line U-937 [5,6] are immunologically related and occur in virtually identical forms. They are denoted as placenta-type PAIs or type-2 PAIs (PAI-2) and differ in immunological, protein chemical [7] and inhibitory properties from type-1 PAIs (PAI-1), i.e. endothelial cell-type PAIs.

During the reaction between urokinase-type (u-PA) or tissue-type plasminogen activators with PAIs, an enzyme-inhibitor complex is formed and then the inhibitor is cleaved; the complex can be dissociated under alkaline conditions. The various stages of the reaction have been observed by means of SDS-PAGE for both type-1 PAI [8] and type-2 PAI [4,6]. For PAI-1 the sequence around the cleavage site was determined by protein chemical methods [9]. Here, a novel, reversed-phase HPLC-based procedure has been employed to monitor the appearance and disappearance of the components in the reaction between PAI-2 and high- and low-M_r u-PAs. PAI-2 interacts in analogous ways with high- and low-M_r u-PAs, even though the latter lacks a large N-terminal part of the structure as compared to the high-M_r form [1]. The cleavage

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Abbreviations: PAI-1 (-2), type-1 (-2) plasminogen activator inhibitor; u-PA, urokinase-type plasminogen activator; HPLC, high-performance liquid chromatography; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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site in PAI-2 was determined by protein sequence analysis.

2. EXPERIMENTAL

2.1. *Materials*

PAI-2 was isolated from human placenta and the human histiocytic lymphoma cell line U-937 as described [4,6]. Pure high- and low-M_r u-PAs (100000 and 180000 IU/mg, respectively) were a gift from Serono (Freiburg, FRG).

2.2. *Reaction between u-PA and PAI-2*

For complex formation solutions of 0.05 mg PAI-2 preparations in 0.15 ml of 0.05 M sodium phosphate, pH 7.2, were mixed with equimolar amounts of high- or low-M_r u-PA in 0.05 ml of 0.1 M NaCl and allowed to incubate for 15 min at room temperature. For complex dissociation 0.2 ml of 1 M ammonia was added and the samples left for 15 or 60 min at 37°C. All reactions were stopped by addition of 0.5 vol. of 70% formic acid before HPLC analysis and SDS sample buffer before SDS-PAGE analysis, respectively.

2.3. *Cyanogen bromide cleavage*

About 0.15 mg PAI-2 was dissolved in 0.1 ml of a solution containing 10% (w/v) CNBr in 70% formic acid. After 2 h at room temperature excess reagents were removed by a stream of N_2 [10].

2.4. *High-performance liquid chromatography*

Separations were performed on an LKB HPLC system using a Nucleosil 300-10 C_{18} reversed-phase column (25 × 0.46 cm, Macherey-Nagel, Düren, FRG). For elution linear gradients between 5 and 70% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid were employed at 1%/min and a flow rate of 1 ml/min at room temperature [11].

2.5. *Polyacrylamide gel electrophoresis*

Electrophoretic analysis [12] was carried out in 0.1 × 10 × 10 cm vertical slab gels containing 0.1% SDS, 10% polyacrylamide and no mercaptoethanol (apparatus from Biometra).

2.6. *Amino acid sequence analysis*

Sequence determination was performed by the Edman degradation method in a prototype spinning-cup sequenator [13].

3. RESULTS AND DISCUSSION

The reaction between high-M_r u-PA and PAI-2 was analysed using an established method [4,6], SDS-PAGE (fig.1), and a novel procedure, reversed-phase HPLC (figs 2,3), in parallel. The results obtained with both methods were in good agreement. The SDS-PAGE system gave additional information about the molecular size of the components, but those components with the lowest

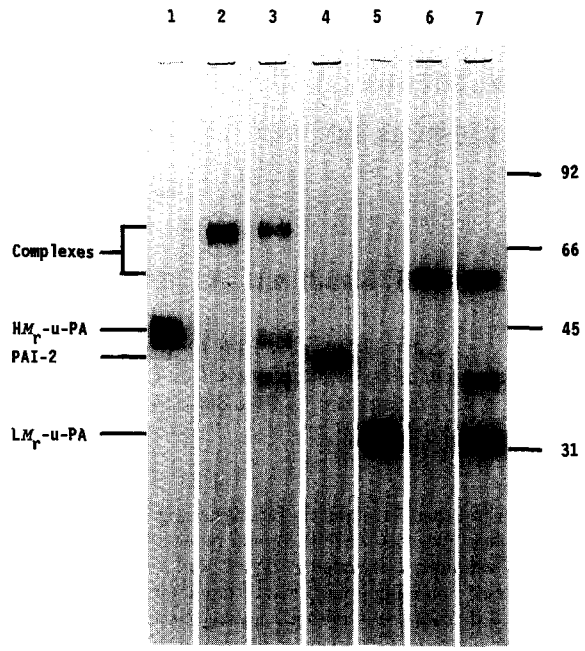

Fig.1. Reaction between PAI-2 and u-PA monitored by non-reducing 10% SDS-PAGE; staining with Coomassie blue. Starting material: PAI-2 (lane 4), high-M_r u-PA (lane 1), low-M_r u-PA (lane 5). Complexes with: high-M_r u-PA (lane 2), low-M_r u-PA (lane 6). Partly dissociated complexes with: high-M_r u-PA (lane 3), low-M_r u-PA (lane 7). M_r values ($\times 10^{-3}$) are indicated on the right.

M_r values could not be detected. The advantages of the HPLC system lie in the facts that the components separated directly could be submitted to protein chemical identification, and furthermore, that all components, even those of low-M_r could be observed and quantitated.

For the activator-inhibitor reaction equimolar amounts of u-PA and PAI-2 were mixed. Complexes sufficiently stable to allow SDS-PAGE or HPLC analysis were formed. The M_r of the complex with high-M_r u-PA appeared to be 82000 and that with low-M_r u-PA 62000 (fig.1), which is somewhat lower than expected from the sum of the components' M_r values, i.e. about M_r 54000 and 33000 for the u-PAs and M_r 45000 for PAI-2. On HPLC analysis components with retention times intermediate between those of the u-PAs and PAI-2 appeared (figs 2,3). Complete complex formation had occurred as judged by the absence of free activators or inhibitor. HPLC analysis clearly demonstrated that at this stage a low-M_r compo-

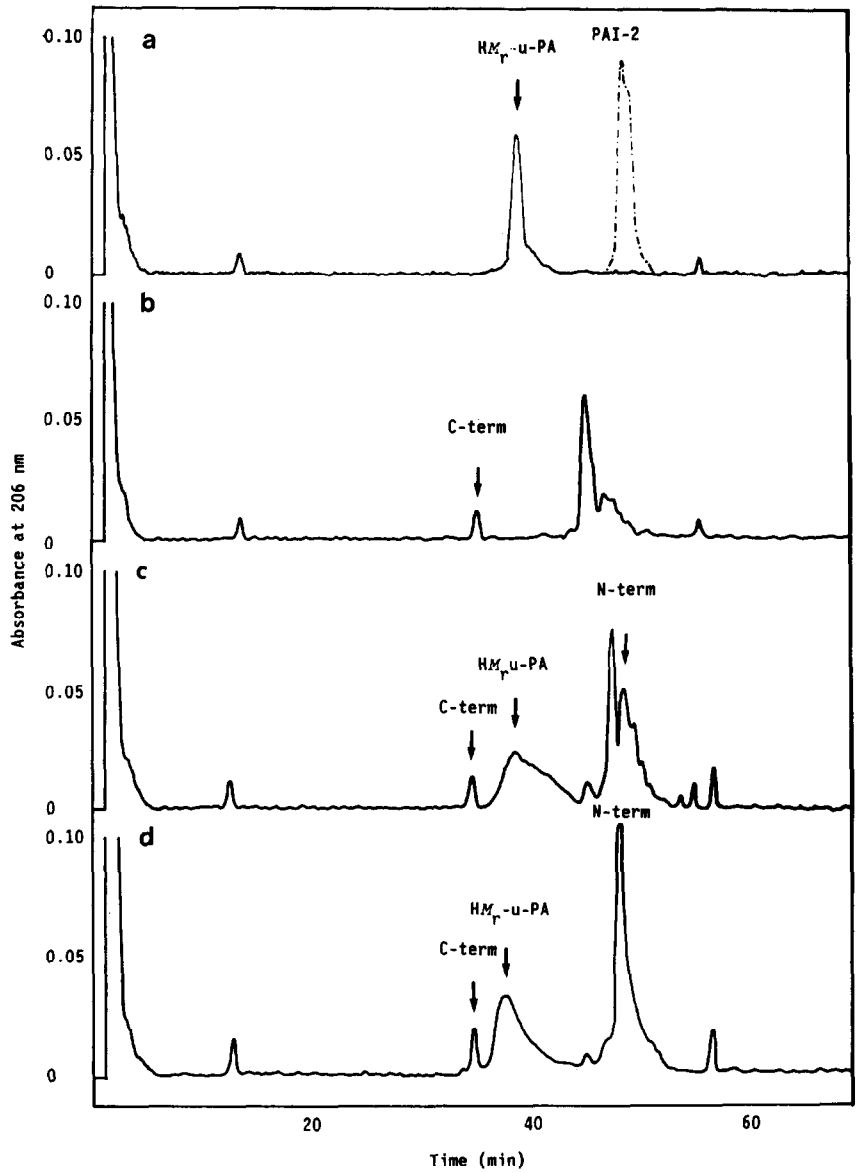

Fig.2. Reaction between PAI-2 and high-M_r u-PA monitored by reversed-phase HPLC. (a) Starting materials (separate, superimposed HPLC analysis), (b) complex formation, (c) partial and (d) complete dissociation.

nent had already been released (figs 2,3, panels b, peak labelled C-term).

The u-PA–PAI-2 complexes were dissociated by incubation with ammonia. After 15 min partial dissociation and after 60 min complete dissociation had taken place under the conditions used. On SDS-PAGE and HPLC analysis components corresponding in M_r and retention time, respectively, to the original u-PA type used in the experiment reappeared (figs 1–3). Unmodified PAI-2 was not recovered as judged by PAGE analysis, but instead a new component with an M_r value approx. 4000 lower appeared. On HPLC analysis, however, this component showed a similar retention behaviour to unmodified PAI-2 (figs 2,3, panels c,d, peak labelled N-term). The relative amount of the low-

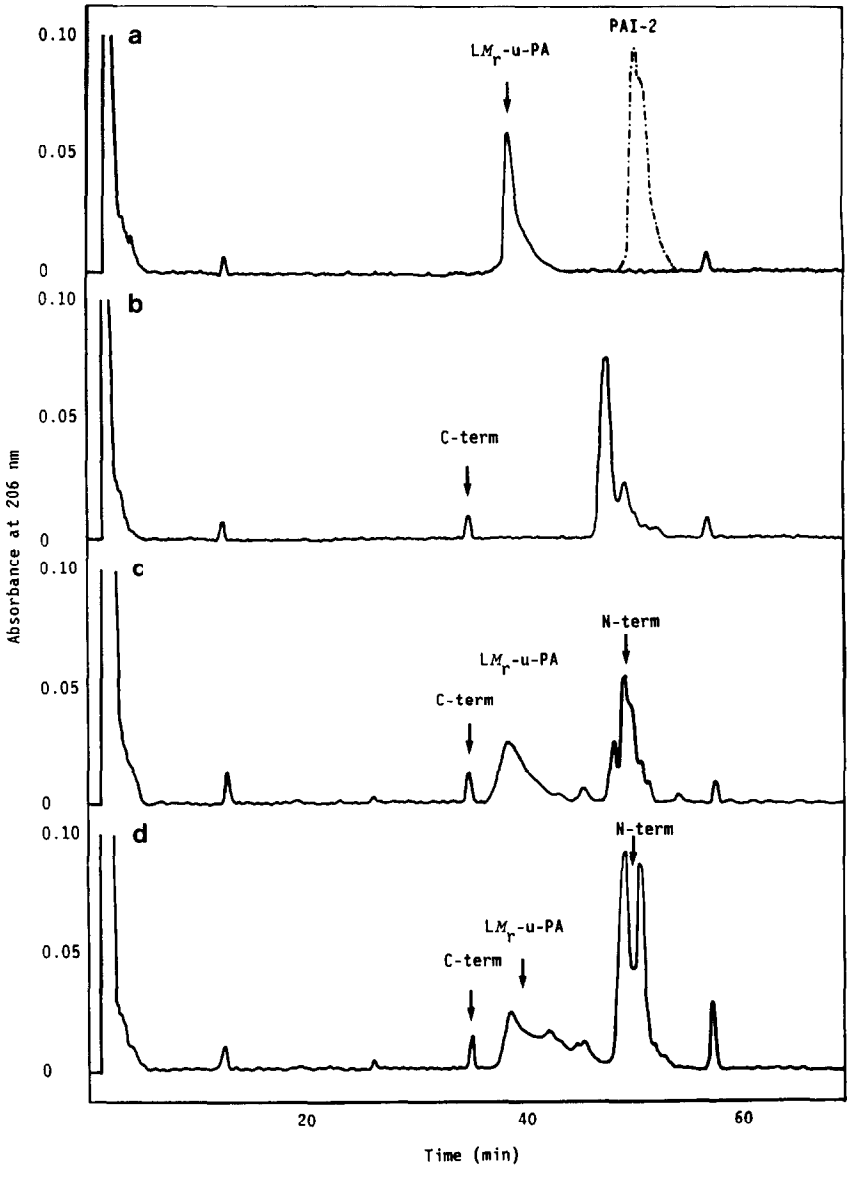

Fig.3. Reaction between PAI-2 and low-M_r u-PA monitored by reversed-phase HPLC. (a) Starting materials (separate, superimposed HPLC analysis), (b) complex formation, (c) partial and (d) complete dissociation.

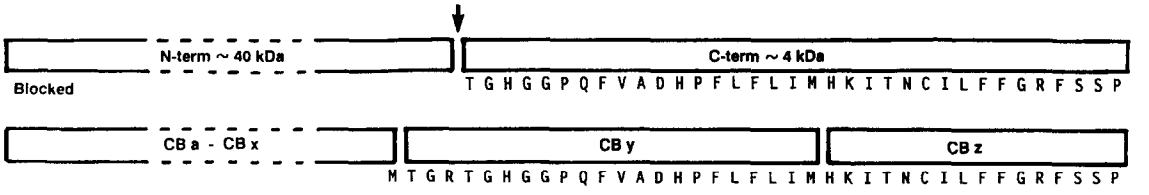

Fig.4. Amino acid sequence around PAI-2 cleavage site (↓) determined by direct sequence analysis of fragments obtained with u-PA (top) and CNBr (bottom) cleavage, respectively.

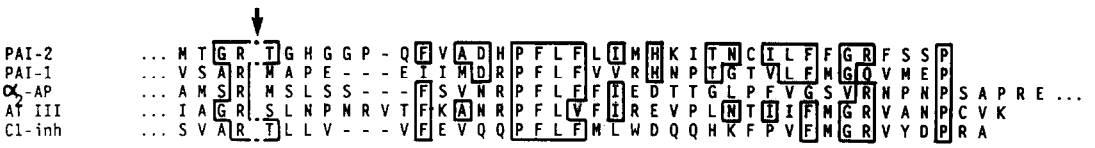

Fig.5. Alignment of PAI-2 cleavage site (↓) sequence with the corresponding sequences of some other Arg-serpins, i.e. endothelial cell-type PAI (PAI-1) [9], α_2-antiplasmin (α_2-AP) [14], antithrombin III (AT III) [15], C1-inhibitor (C1-inh) [16]. Identical residues in PAI-2 and other serpins in corresponding positions are boxed.

M_r HPLC component was not influenced by dissociation (figs 2,3, peak labelled C-term).

PAI-2 and the new components recovered after the reaction were subjected to N-terminal amino acid sequence analysis. Neither PAI-2 itself nor the component with the similar retention time gave rise to any sequence. However, the low-M_r HPLC component showed a sequence starting with Thr-Gly-His-Gly-Gly- (fig.4, top). From these results, it was concluded that the low-M_r component corresponds to the C-terminal part and the N-terminally blocked, high-M_r component to the N-terminal part of PAI-2, the M_r values being about 4000 and 40000, respectively.

In an independent experiment PAI-2 was cleaved with CNBr after the Met residues, and then the fragments were separated by reversed-phase HPLC (not shown) and characterized by N-terminal sequencing. A 22-amino-acid residue fragment showed a sequence starting with Thr-Gly-Arg-Thr-Gly-His-Gly-, i.e. it contained the N-terminal part of the peptide released on reaction of PAI-2 with u-PA (fig.4, bottom). A 16-residue fragment starting with His-Lys-Ile-Thr-, was devoid of Met (or homoserine) in the composition, and therefore corresponds to the C-terminus of PAI-2 (not shown). From further sequence and amino acid analysis data, it was clear that the C-terminal peptide derived from the reaction with u-PA is composed of the final 19 residues of the former CNBr fragment and the complete fragment of the latter, i.e. the peptide contains 35 amino acid residues. This result is in good agreement with the decrease of 4000 in the M_r value of PAI-2 during the reaction (fig.4). The sequence of the penultimate CNBr fragment also provided the reactive site sequence of PAI-2. A single Arg–Thr bond is cleaved during the reaction with the plasminogen activator, the cleavage of an arginyl bond being in agreement with the expected substrate specificity of u-PA. Identical results were obtained throughout with PAI-2 preparations from the two sources, human placenta and the human monocyte-like cell line U-937.

The sequence around the cleavage site in PAI-2 is aligned with the corresponding sequence of other serine proteinase inhibitors in which an arginyl bond is cleaved (fig.5), i.e. other Arg-serpins [9,14–17]. Within the short sequences compared only a few completely invariant amino acid residues are found in addition to the Arg at the cleavage site. It is noteworthy that the C-terminal peptide of PAI-2 is released on reaction with u-PA in the absence of a reducing agent even though this peptide contains Cys which might otherwise have been expected to disulfide-link the C-terminal and N-terminal parts of PAI-2 in an analogous way as these parts are linked in antithrombin III [15].

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