IRREVERSIBLE INHIBITION AND PEPTIDE MAPPING OF URINARY PLASMINOGEN ACTIVATOR UROKINASE

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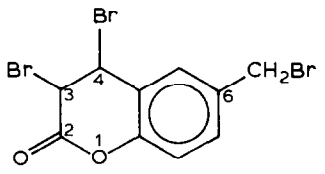
1. Introduction

The activation of plasminogen by trypsin-like plasminogen activators constitutes a widespread mechanism for generating localized extracellular proteolysis [1]. Plasminogen activators exist in several molecular forms that can be distinguished on the basis of their M_r-values and their susceptibility to inhibition by antibodies against the normal urinary enzyme, urokinase [2]. Differences between tissue and urinary activators concerning their binding affinities towards certain synthetic substrates [3] and their susceptibility to inactivation by peptidyl chloromethylketone inhibitors [4] have also been reported. The nature of the relationship that exists between the multiple forms of plasminogen activators (products of different genes or forms derived by partial proteolysis of a common precursor) is of constant concern. Peptide maps of these proteins would contribute to answer this question unequivocally.

The well-characterized human urinary plasminogen activator, urokinase (EC 3.4.99.26) has been chosen in this study and its peptide map is presented. To identify the peptides containing the amino acid residues of the active site, peptide maps of the enzyme modified by 3,4-dihydro-3,4-dibromo-6-bromomethylcoumarin (scheme) are reported. This bifunctional reagent possesses a reactive ester bond with a *cis* configuration and an alkylating function which appears after hydrolytic cleavage of the ester bond [5]. Some proteases have been reported to be inactivated by this reagent [6,7]: histidine-57 was preferentially modified by the activated alkylating function generated in the active site of α-chymotrypsin [8].



3,4-dihydro-3,4-dibromo-6-bromomethyl coumarin

2. Materials and methods

Human urokinase (RFU 810) was a kind gift from Choay, France. Its concentration in solution was determined by the amido black procedure [9] using bovine serum albumin as standard. Active site titrations were carried out with *p*-nitrophenyl-*p'*-guanidinobenzoate as in [10]. Sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis was performed using 12.5% polyacrylamide slab gels according to [11]. Gels were scanned with a Joyce densitometer at 500 nm. The 2 forms of urokinase (47 000 and 33 000 M_r) were ~90–95% and 5–10%, respectively. Their plasminogen activator activity was detected by electrophoretic analysis in SDS–polyacrylamide gels containing copolymerized plasminogen and gelatin [12]. The final concentrations were: 0.125 M Tris (stacking gel), 0.05% (w/v) gelatin and 40 μg human plasminogen/ml. The activity was nearly exclusively localized in a band of app. M_r 47 000.

Pepsin was a twice-crystallized product, purchased from Worthington Biochemicals; α-CBZ–L-lysine–*p*-

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nitrophenylester and 6-methylcoumarin were obtained from Sigma and Schuchardt, respectively. Standard chemicals were from Merck, Fluka and Sigma.

2.1. *Preparation of 3,4-dihydro-3,4-dibromo-6-bromomethylcoumarin*

The reagent was prepared and characterized as in [6]. The radioactive 3,4-dihydro-3,4-dibromo-6-bromo-[^{3}H]methylcoumarin synthesized according to [13] was obtained with a spec. act. 5.5 μCi/mmol.

2.2. *Kinetic analysis*

The esterase activity of the enzyme derivatives was determined towards α-CBZ–L-lysine–*p*-nitrophenyl ester according to [14] using a Cary model 15 spectrophotometer. For native enzyme, the conditions employed were: 7.4×10^{-8} M enzyme, 0.1 M sodium phosphate buffer (pH 6.8), 0.1 M NaCl, 2×10^{-4} M α-CBZ–L-lysine–*p*-nitrophenyl ester, 25°C. The increase in absorbance was monitored at 360 nm using $\epsilon = 6.6 \times 10^3\ M^{-1} \cdot cm^{-1}$ [15]. The initial velocities were corrected for the spontaneous hydrolysis of the ester. All kinetic data were calculated on the basis of molarity of active sites, or percentage of active enzyme, as determined by active site titration.

2.3. *Reactions with 3,4-dihydro-3,4-dibromo-6-bromomethylcoumarin*

2.3.1. Kinetics of inactivation

The following general protocol was employed for the determination of the rate of enzymatic inactivation. At time zero, 3,4-dihydro-3,4-dibromo-6-bromomethylcoumarin (final conc. 9.5–147 μM) was added in 10 s to a solution of urokinase (4.9 μM) in a 0.1 M sodium phosphate buffer, 0.1 M NaCl (pH 6.8) at 4°C or 25°C. Aliquots (15 μl) were removed at intervals and assayed for remaining enzymatic activity by addition to 1 ml appropriate standard assay mixture (see above). The apparent pseudo-first-order rate constants k_{app} were obtained from least-squares analysis of semilog plots of % remaining activity against time.

2.3.2. Estimation of bound ^{3}H-labelled groups

Urokinase (0.7 nmol) was treated for 10 min with a 20-fold molar excess of 3,4-dihydro-3,4-dibromo-6-bromomethyl[^{3}H]coumarin under the above conditions (25°C). The reaction mixture was immediately filtered through a 0.2 μm cellulose acetate membrane (Schleicher and Schüll) whose radioactivity was measured. In some experiments, the enzyme was incubated in the presence of 18 μM *p*-nitrophenyl-*p'*-guanidinobenzoate before treatment with the labelled reagent.

2.4. *Peptide maps*

A sample of 0.7 mg native or modified urokinase (treated with the labelled reagent as in section 2.3.2) was filtered through a cellulose-acetate membrane and washed extensively. The enzyme on the membrane was digested with pepsin (0.07 mg) in 0.4 ml 5% formic acid for 18 h at 37°C. After adjustment to pH 5 with pyridine, the reaction mixture was lyophilized. Aliquots containing ~70 μg original urokinase in 1–2 μl pyridine–water (50:50) were spotted onto a 20 × 20 cm thin-layer chromatogram sheet (cellulose, F1440, Schleicher and Schüll). Chromatograms were carried out for 3 h in *n*-butanol–pyridine–acetic acid–water, 30:20:6:24 (by vol.). The plates were then exposed for 2 h at room temperature to performic acid vapors [16]. After drying, electrophoresis in the second dimension was done at 400 V for 1.5 h at pH 2 (2% formic acid–8% acetic acid, v/v) using a double chamber Desaga apparatus. The peptides were detected with the cadmium–ninhydrin reagent [17] or with 0.01% (w/v) fluorescamine in acetone (after spraying with 5% (v/v) triethylamine in acetone) [18] and those containing histidine with the Pauly reagent [19]. Labelled peptides were located by fluorography (EN^{3}HANCE^{TM}Spray, New England Nuclear; Kodak X-Omat X-Ray Film, exposures from 4–10 days after flash activation [20]). Any background radioactivity due to residual reagent or to its hydrolysis products was determined by peptide mapping in similar conditions.

3. Results

3,4-Dihydro-3,4-dibromo-6-bromomethylcoumarin is a time-dependent inactivator of urokinase (fig.1). The residual activity observed at the end of the reaction depends on the molar ratio of inhibitor to enzyme. With a 30-fold molar excess, the activity of urokinase was inhibited >90% of the initial level within 3 min. In all cases, the activity losses followed pseudo-first-order kinetics characterized by the rate constants k_{app}. These rate constants were proportional to the inhibitor concentration at low concentrations (fig.1) and displayed a tendency to level off

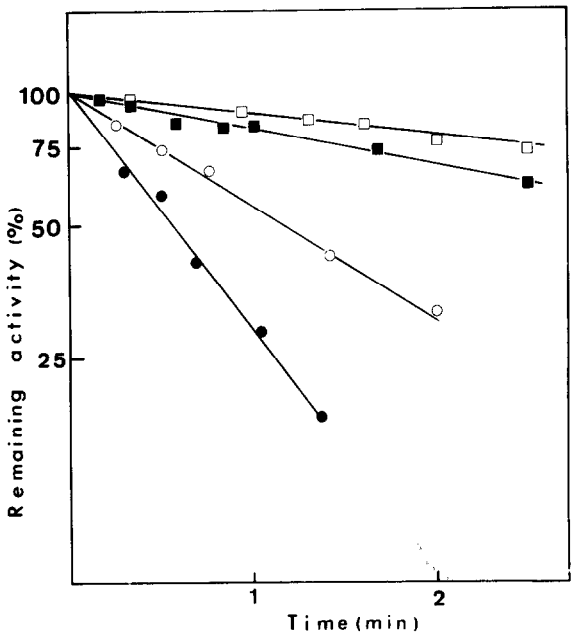
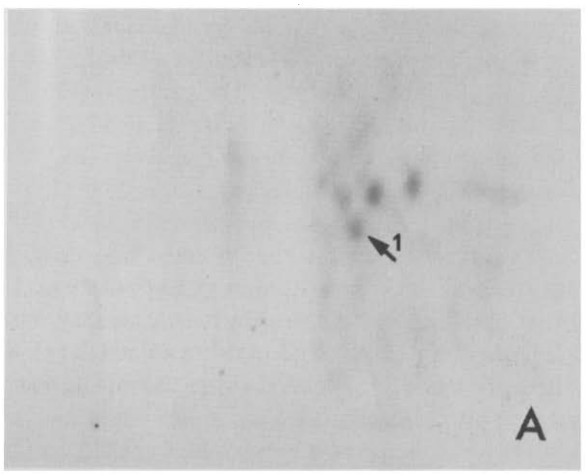
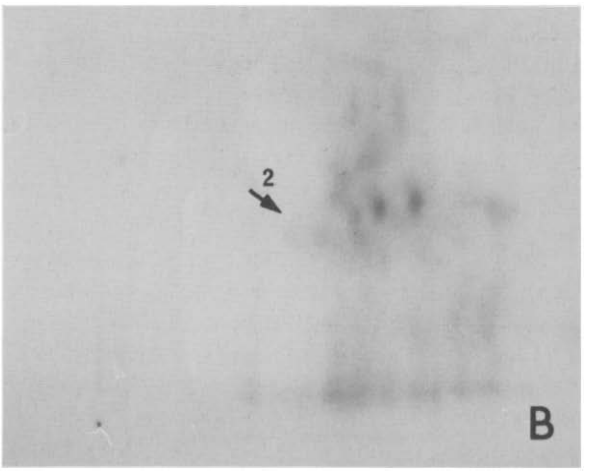


Fig.1. Kinetics of inactivation of urokinase (4.9 μM) by 9.0 μM (□), 15.2 μM (■), 49.8 μM (○) and 99.6 μM (●) 3,4-dihydro-3,4-dibromo-6-bromomethylcoumarin at pH 6.8 in 0.1 M phosphate buffer and 0.1 M NaCl at 25°C. The activity was determined against α-CBZ–L-lysine–*p*-nitrophenylester at pH 6.8 and 25°C for native and treated enzyme. The activity of the native enzyme was taken equal to 100.

at higher concentrations (not shown). At 25°C, the experimental second-order rate constant obtained by dividing k_{app} by the inhibitor concentration is equal to $1.25 \times 10^4\ M^{-1}.min^{-1}$ ($k_{app}/[I] \sim 3.2 \times 10^2\ M^{-1}.min^{-1}$ at 4°C). No reactivation was observed after an incubation period of 8 h at 4°C. In the same conditions, the spontaneous loss of activity of a control sample was ⩽10%. It was verified that the hydrolysis products of the reagent are inactive towards urokinase.





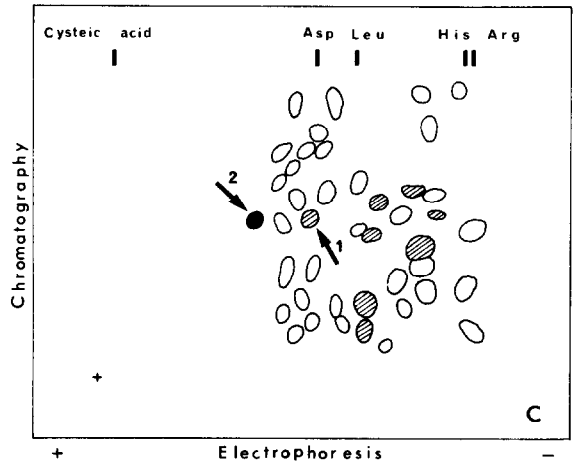


Fig.2. Two-dimensional peptide maps: (A,B) peptide maps after ninhydrin staining of native (A) and modified (B) urokinase; (→1 and →2) the peptide which disappeared and the peptide which appeared upon reaction with 3,4-dihydro-3,4-dibromo-6-bromomethylcoumarin, respectively; (C) tracing of maps. Peptides containing histidine are hatched. The black spot represents the radioactive peptide. The positions of cysteic acid, aspartate, leucine, histidine and arginine after electrophoresis are indicated.

Incubation experiments with the reagent labelled in the moiety expected to undergo covalent attachment to the enzyme showed that 1.2 ± 0.1 equiv. [^{3}H]alkylating group/mol active enzyme was incorporated. When the enzyme had been previously completely inhibited by *p*-nitrophenyl-*p'*-guanidinobenzoate which reacts with the active serine [21], a negligible incorporation of the labelled reagent was observed (~0.1 equiv./mol protein).

The peptide maps of native and modified urokinase visualized by ninhydrin staining are depicted in fig.2. The main difference between both fingerprints lies in the disappearance (native urokinase) of a Pauly- and ninhydrin-positive peptide (fig.2, →1), and the appearance (modified urokinase) of a new peptide (fig.2, →2) which was ninhydrin-positive and radioactive. Indeed, the autoradiogram of the peptide map obtained with urokinase modified by the labelled reagent displayed a unique spot which was superimposed on the acidic peptide appearing after treatment with the reagent (fig.2, →2). No trace of radioactive material corresponding to the residual labelled reagent or hydrolysis products was detected.

4. Discussion

The inactivation of urokinase by 3,4-dihydro-3,4-dibromo-6-bromomethylcoumarin meets the criteria for active-site-directed, covalent enzyme inactivation [22] as supported by kinetic data, protection by *p*-nitrophenyl-*p'*-guanidinobenzoate against inactivation and stoichiometry of labelling by the radioactive reagent. Consequently, the main part of the inhibition is performed when the active serine is intact, suggesting a minimal reaction scheme analogous to that in [6,8] for the inactivation of α-chymotrypsin. After formation of the Michaelis complex, the enzyme is acylated on the active serine; in the transient acyl-enzyme, the reactive *p*-hydroxybenzylhalide is transformed into quinone methide which is instantaneously attacked either by any nucleophilic enzymic residue located in the vicinity (thus leading to covalent modification) or by medium water (in this case, the enzyme is restored after deacylation). This scheme agrees with the alkylation of a nucleophilic residue within (or near) the active site. The value obtained for $k_{app}/[I]$ is smaller than that estimated for α-chymotrypsin [6]. Affinity labelling by peptides or arginine chloromethyl ketone are less effective [23].

By peptide mapping, a typical position of the acidic peptide carrying the alkylated amino acid residue was accurately established (fig.2, →2). Its alkylation is supported by the following facts:

(i) It is the only radioactive peptide after inactivation by the tritiated reagent;
(ii) Its position near the cathode agrees with the possible hydrolysis of the ester bond with active serine during peptide map treatments thus generating a negatively charged carboxylate group.

The alkylated peptide replaces a Pauly-positive peptide present in the peptide map of native urokinase (fig.2, →1). This last result suggests that a histidine residue in (or near) the active site might be modified. Indeed, urokinase is a serine protease [21] which can be related to serine proteases that depend upon the Asp ... His ... Ser triad for their catalytic action since direct evidence of a histidine residue in the active center was obtained [24]. It is noteworthy that both peptides 1 and 2 (fig.2) are located in a more acidic position than the corresponding peptides of native and modified trypsin examined in the same conditions (not shown).

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