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Proteolytic Modification of Tissue Plasminogen Activator: Importance of the N-Terminal Part of the Catalytically Active B-Chain for Enzymatic Activity†

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ABSTRACT: Native one-chain tissue plasminogen activator (t-PA) was rapidly converted to the two-chain form by trypsin–Sepharose cleavage. This caused an increase in the amidolytic activity on low molecular weight peptide substrates, while plasminogen activation in the presence of fibrin markedly decreased. Cleavage sites were identified by N-terminal sequence analysis of reduced and carboxymethylated peptides. In the B-chain, the expected cleavage at Arg_{278}–Ile_{279} was identified. Furthermore, a specific cleavage site was found at Arg_{302}–Ser_{303}, 24 amino acids from the N-terminus of the B-chain. The peptide released by this cleavage (designated B_{1-24}) remained associated with the activator molecule by strong noncovalent interactions but could be dissociated under denaturing conditions (4 mol/L of guanidine hydrochloride), leading to a 20-fold decrease in amidolytic activity. Addition of purified B_{1-24} peptide to t-PA treated in this manner restored the activity in a concentration-dependent way. In contrast to trypsin, cleavage of the single-chain t-PA molecule with endoproteinase Lys-C generated a two-chain form of the activator, without simultaneous increase in the amidolytic activity. By sequence analysis, a major cleavage was identified at Lys_{280}–Gly_{281}, two residues into the B-chain. Together, the results presented provide additional information on the one-chain to two-chain conversion of t-PA and the role of the free N-terminus of the B-chain.

Plasminogen activators catalyze the activation of plasminogen to the active proteolytic enzyme plasmin. Plasmin in turn degrades fibrin clots to soluble components. Tissue plasminogen activator is sythesized in the endothelial cells as a single-chain molecule but can be converted to a two-chain form by plasmin cleavage (Wallén et al., 1982, 1983). The two chains, held together by a disulfide bond, are designated A-chain, for the polypeptide originating from the N-terminal part of the enzyme, and B-chain, for the polypeptide from the C-terminal part of the enzyme. The primary structure of t-PA[1] has been determined by both protein and cDNA sequence analysis (Pennica et al., 1983; Pohl et al., 1984). For the protease domain, extensive homologies with other proteases have been shown (Strassburger et al., 1983).

Recently, several studies on structure–function relationships in the t-PA molecule using molecular biology techniques such as deletion mutants (van Zonneveld et al., 1986) or site-specific

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[1] Abbreviations: AA, amino acid(s); DFP, diisopropyl fluorophosphate; DTE, dithioerythritol; NaCl/P_i, phosphate-buffered saline (50 mM phosphate, pH 7.3, containing 0.1 g/L Triton X-100, I= 0.15); PAGE, polyacrylamide gel electrophoresis; pNA, *p*-nitroanilide; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; t-PA tissue plasminogen activator.

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mutations (Petersen et al., 1988; Tate et al., 1987) have been published. From the latter studies, it can be concluded that the single-chain t-PA molecule has a substantial enzymatic activity. In the present paper, the effects of trypsin cleavage of single-chain t-PA have been studied, with special emphasis on the N-terminal part of the B-chain and its role in the activity of the B-chain. Furthermore, the role of the lysine residue at position 2 in the B-chain (Lys_{280}) has been studied by proteolysis of native one-chain t-PA with endoproteinase Lys-C.[2]

Materials and Methods

Proteins. Native one-chain tissue plasminogen activator (t-PA) (Wallén et al., 1983; Norrman et al., 1985), two-chain t-PA, plasminogen, and plasmin (Wallén et al., 1982), and desAA fibrinogen monomers (Norrman et al., 1985) were all prepared as described. t-PA was labeled with ^{125}I according to the iodogen method (Salacinski et al., 1981; Norrman et al., 1987). N^{α}-*p*-Tosyl-L-phenylalanine chloromethyl ketone treated trypsin (Sigma) and plasmin were coupled to Sepharose 4B (Pharmacia) by the CNBr method (Axén et al., 1967). Incorporation of proteins as determined by amino acid analysis after acid hydrolysis was found to be 2.6 mg/g for plasmin–Sepharose and 1.5 mg/g for trypsin–Sepharose. Endoproteinase Lys-C from *Lysobacter enzymogenes* was purchased from Boehringer Mannheim.

Proteolytic Treatment and Enzymatic Assays. For digestions with trypsin, 7 mg of trypsin–Sepharose was added per 100 μg of t-PA dissolved in 0.15 mol/L of ammonium bicarbonate to a final activator concentration of 100 μg/mL (1.7 μmol/L). The reaction mixture was incubated during continuous end-over-end rotation at ambient temperature. After 1, 2, and 24 h, the incubation vessel was centrifuged, and a sample was taken and filtered through a 0.22- or 0.45-μm filter (HV 4, Millipore).

Digestion with endoproteinase Lys-C was performed by adding 685 milliunits (23 μg) of the enzyme to 137 μg of t-PA dissolved in 1 mL of 0.15 mol/L of ammonium bicarbonate. After incubation at ambient temperature (4 and 24 h), the reaction was terminated by quick freezing. Subsequent plasmin treatment of lysylprotease-digested t-PA was performed by incubating 190-μL aliquots of the respective digestion mixtures with 1 mg of plasmin–Sepharose. At regular time intervals, samples were removed, diluted 1:100 with $NaCl/P_i$, and filtered through a 0.45-μm filter. The amidolytic activity was determined as described below.

Amidolytic activity of t-PA was determined with the substrate D-Ile-Pro-Arg-pNA (KABI S-2288) at a final concentration of 0.4 mM (Norrman et al., 1985, 1986; Rånby et al., 1983). For kinetic analysis, a substrate concentration in the range 0.1–2 mM was used. Plasminogen activation activity was determined as described (Rånby et al., 1982c; Norrman et al., 1985, 1986), using the substrate D-Val-Phe-Lys-pNA (Kabi S-2390). The presence of trace amounts of endoproteinase Lys-C did not influence the results.

Binding to Arginine–Sepharose and Fibrin. Affinity of ^{125}I-labeled, trypsin-digested activator to arginine–Sepharose was determined by using a 5-mL column of arginine–Sepharose, equilibrated in 0.5 M ammonium bicarbonate, containing 0.1% Tween 80. Samples were diluted in the same buffer to 8 μg/mL. The column was washed with the equilibration buffer and eluted with 2 M guanidine hydrochloride (BRL ultrapure) containing 0.1% Tween. Radioactivity was measured in a LKB-Wallac minigamma counter.

Fibrin binding was determined by mixing fibrinogen (final concentration 1 μM), aprotinin (final concentration 5 KIE/mL), and ^{125}I-labeled t-PA (final concentration 0.1 μM) in nitrocellulose tubes. The volume was adjusted to 270 μL with 50 mmol/L of sodium phosphate buffer, pH 7.3, containing 100 mmol/L of ammonium bicarbonate, 0.5 g/L pluronic F68, and 0.01 g/L Tween 80). Thrombin (30 μL, final concentration 0.3 NIH/mL) or buffer was added, and the tubes were incubated for 4 h at room temperature and subsequently centrifuged for 30 min, ~30000*g* at +4 °C in a Beckman ultracentrifuge using a SW 65 swing-out rotor. From the supernatant, 50 μL was removed, whereafter the radioactivity in this aliquot and in the rest of the tube was determined and the amount of bound t-PA calculated.

Sephadex G-50 Size Exclusion Chromatography. Trypsin-treated t-PA was gel filtrated on 25 × 2.5 cm columns of Sephadex G-50 superfine (Pharmacia), equilibrated in either 0.5 mol/L of ammonium bicarbonate containing 0.1 g/L of Tween 80 or 0.1 mol/L of Tris-HCl buffer, pH 8.15, containing 4 mol/L of guanidine hydrochloride. The absorbance at 280 nm was measured, and the high molecular weight fractions from the guanidine hydrochloride column were pooled and desalted on a 22 × 2.5 cm column of Sephadex G-25 fine, equilibrated in 0.5 mol/L of ammonium bicarbonate containing 0.1 g/L of Tween 80. From this material, a sample was taken for RP-HPLC. The amidolytic activity against the substrate S-2288 was determined after dilution 1:10–1:100. As control experiments, two-chain t-PA was chromatographed under the same conditions.

Electrophoretical and Structural Analysis. Lyophilized aliquots of samples from digestion experiments were reduced and carboxymethylated for electrophoresis or HPLC. Samples were dissolved in 250 μL of buffer (0.1 M Tris-HCl, pH 8.15, containing 6 M guanidine hydrochloride and 10 mM EDTA), and DTE (1 μL, 0.5 mol/L) in molar excess over the cysteine residues was added. After reduction for 60 min at 60 °C, the samples were carboxymethylated with either 1.5 μL of 0.5 mol/L of iodoacetate or 15 ML of 50 mmol/L of iodoacetate containing 20% [^3H]iodoacetate (Amersham).

Samples were desalted on PD-10 columns (Pharmacia) equilibrated with 30% (5 mol/L) acetic acid. The acetic acid was evaporated in a Savant Speed-Vac vacuum centrifuge, and the samples were dissolved in 35 μL of electrophoresis sample buffer (0.06 M Tris buffer, adjusted to pH 6.8 with HCl and containing 12% glycerol, 1.2% SDS, and 0.1% bromophenol blue dye). SDS–polyacrylamide gel electrophoresis was performed in 10% acrylamide mini slab gels by using a Laemmli system (Laemmli, 1970). The gels were run at 200-V constant voltage for 45 min and stained with 0.2% Coomassie Brilliant Blue in acetic acid/methanol/water (10/40/50). In some cases, samples were active site labeled with [^3H]DFP prior to electrophoresis (Norrman et al., 1985).

HPLC was performed with a high-pressure gradient system (LKB) using a VYDAC 218TP54 wide-pore C_{18} column. The equilibration buffer was 0.1% (13 mmol/L) TFA (Rathburn, sequence grade), and peptides were eluted by using a gradient (2–60%) of acetonitrile (HPLC quality, J. T. Baker) containing 0.08% (10 mmol/L) TFA. The absorbance at 215 nm was measured, and peptides were manually collected. The radioactivity in each peak was determined by liquid scintillation counting, using LKB Optiphase Highsafe scintillation cocktail.

The N-terminal amino acid sequence of peptides was determined by using an Applied Biosystems 477A liquid-phase

[2] Enzymes: trypsin, EC 3.4.21.4; plasmin, EC 3.4.21.7; tissue plasminogen activator, EC 3.4.21.31; endoproteinase Lys-C, EC 3.4.21.50.

Table I: Enzymatic Activity of t-PA after Trypsin–Sepharose Cleavage

time (h)	amidolytic activity in absence of fibrin[a]	plasminogen activation in the absence of fibrin[a]	plasminogen activation in the presence of fibrin[a]
0	100	100	100
1	727 ± 140	246 ± 66	87 ● 22
4	741 ● 125	110 ± 40	70 ± 37
24	717 ● 165	79 ● 40	13 ± 5

[a] Percent of starting value ± SD. Mean of five different trypsin treatment experiments analyzed in duplicates.

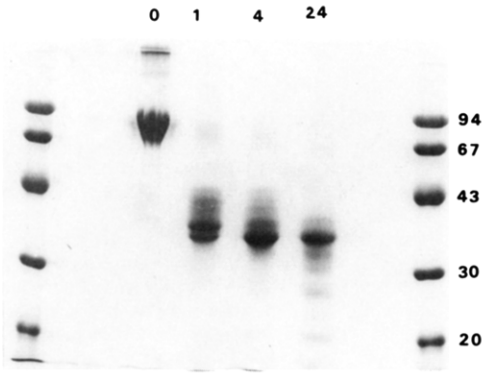

FIGURE 1: SDS–PAGE analysis of trypsin–Sepharose-digested t-PA. Samples of trypsin-digested t-PA were reduced and carboxymethylated as described under Materials and Methods. The gel was stained with Coomassie Brilliant Blue. Positions of molecular weight markers and digestion times in hours are indicated.

sequencer with an on-line 120A PTH analyzer. Initial yield using β-lactoglobulin as standard was 47%, and the repetitive yield was 97%. Peptides from HPLC were lyophilized and thereafter dissolved in 0.1% TFA prior to application. In some cases, peptide-containing fractions were directly applied on the sequencer filter. The radioactivity in each sequencer cycle was determined by liquid scintillation.

RESULTS

Effects of Trypsin–Sepharose Digestion on Enzymatic Activity and Ligand Binding. Digestion of one-chain tissue plasminogen activator (t-PA) with Sepharose-bound trypsin caused a marked increase in the amidolytic activity (Table I). Electrophoretical analysis after 1 h of trypsin proteolysis demonstrated the two-chain form of t-PA, with the A-chain from the N-terminus and the B-chain from the C-terminus part of the native molecule (Figure 1). The typical A-chain doublet initially seen (Pohl et al., 1987) was extensively degraded after 24 h of trypsin digestion. Furthermore, a proteolytic modification of the active site containing B-chain was indicated by the increased mobility on SDS–PAGE. This has also been indicated by active site labeling of trypsin-treated t-PA with [^{3}H]DFP (Norrman et al., 1986).

The plasminogen activation activity initially increased, followed by a reduction to 80% of the starting value after 24 h (Table I). When analyzed in the presence of fibrin, the plasminogen activation activity decreased continuously. The binding to fibrin decreased rapidly, while the binding to arginine–Sepharose was unaffected after 1 h, but then rapidly fell (Table II).

Purification and Sequence Analysis of Tryptic Peptides. Peptides from reduced and carboxymethylated trypsin digested t-PA were purified by RP-HPLC (Figure 2). The B-chain eluted at 46% acetonitrile, as determined by separate chromatography of B-chain purified by arginine–Sepharose chromatography (Rånby et al., 1982b).

Table II: Binding of Trypsin-Degraded t-PA to Fibrin and Arginine–Sepharose

time (h)	fibrin binding[a]	arginine-Sepharose binding[a]
0	100	100
1	47	100
4	22	45
24	1	1

[a] Percent of starting value.

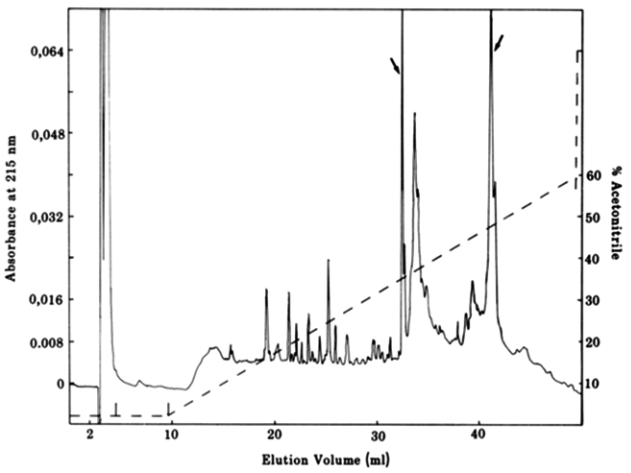

FIGURE 2: Reverse-phase HPLC analysis of trypsin–Sepharose-digested t-PA. About 50 μg of t-PA digested with trypsin for 24 h was lyophilized, reduced, and carboxymethylated as described under Materials and Methods. About 20 μg was injected into the column. Arrows indicate the position of B_{1-24} peptide (left) and degraded B-chain (right), respectively.

The peptide corresponding to the well-resolved peak indicated by the left arrow in the HPLC chromatogram (Figure 2) was collected for N-terminal sequence analysis. More than 300 pmol of peptide was analyzed, and the complete sequence, I-K-G-G-L-F-A-D-I-A-S-H-P-W-Q-A-A-I-F-A-K-H-R-R, was determined without evidence of contaminants. The peptide fragment, which stretches between Ile_{279}[3] and Arg_{302} in native t-PA and contains the first 24 AA N-terminal residues of the B-chain, was denoted B_{1-24}. The B_{1-24} peptide could be identified by RP-HPLC after only 1 h of trypsin digestion and, after 4 h, it was completely cleaved off. Another peptide, which on SDS–PAGE had the same mobility as the modified B-chain, was eluted at 47% acetonitrile (right arrow, Figure 2). The N-terminal sequence of this fragment started with S-P-G-E-, corresponding to Ser_{303} at position 25 in the B-chain.

Most of the peptides eluting at 10–30% acetonitrile (Figure 2) were collected and sequence analyzed. These peptides were found to originate from the N-terminal part of t-PA. After 4 h of trypsin digestion, cleavages were identified after residues Arg_3, Lys_{13}, and Arg_{30}, i.e., in the finger domain. After 24 h, additional cleavages were found after residues Arg_{33}, Arg_{43}, Lys_{52}, and Arg_{58}, i.e., in the EGF domain. At least 4 AA residues were determined in each case.

Importance of the B_{1-24} Peptide for Activity of Trypsin-Digested t-PA. To potentially remove the B_{1-24} peptide, t-PA digested with trypsin–Sepharose for 1, 4, and 24 h was subjected to size exclusion chromatography. After chromatography in 0.5 mol/L of ammonium bicarbonate, the amidolytic activity of t-PA digested with trypsin–Sepharose for 24 h decreased to about 90% of the initial value (Table III).

[3] The amino acid residues of t-PA are numbered according to the longest form of the mature protein (Wallén et al., 1983; Pohl et al., 1984).

Table III: Amidolytic Activity of t-PA before and after Gel Filtrations[a]

time of trypsin–Sepharose digestion (h)	initial amidolytic activity	amidolytic activity after nondenaturing gel filtration (%)	amidolytic activity after denaturing gel filtration (%)
1	53	57 (107)[b]	13 (25)[b]
4	56	49 (88)[b]	3.3 (6)[b]
24	49	44 (90)[b]	2.2(5)[b]
2-chain control	54	NP[c]	38 (71)[b]

[a] Amidolytic activity = absorbance increase·10^3·min^{-1}·μg^{-1}·mL^{-1}, calculated for 1-cm path length at a substrate concentration of 0.4 mmol/L. [b] (*n*) = percent of starting value. [c] NP = experiment not performed.

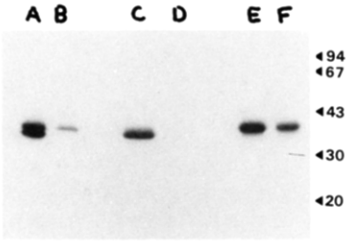

FIGURE 3: Fluorography of [^{3}H]DFP-labeled trypsin–Sepharose-digested t-PA before and after denaturing gel permeation chromatography (DGPC). [^{3}H]DFP incorporation, reduction and carboxymethylation, and electrophoresis were performed as described under Materials and Methods: t-PA digested with trypsin for 1 h before (A) and after (B) DGPC; t-PA digested with trypsin for 24 h before (C) and after (D) DGPC; two-chain t-PA prepared by limited plasmin cleavage before (E) and after (F) DGPC. Positions of molecular weight markers are indicated.

Peptide mapping by RP-HPLC after reduction and carboxymethylation demonstrated that the B_{1-24} peptide remained bound to the t-PA molecule under these conditions. Chromatography in 2 mol/L of guanidine hydrochloride did not dislocate the B_{1-24} peptide, while size exclusion chromatography under more denaturing conditions (4 mol/L of guanidine hydrochloride) removed the B_{1-24} peptide, as shown by RP-HPLC peptide mapping.

After the B_{1-24} peptide had been removed, the amidolytic activity was reduced to about 5% of the initial value (Table III). A kinetic analysis of the 4-h sample showed that k_{cat} decreased 30-fold after removal of the B_{1-24} peptide, while the K_m decreased 4-fold. Control experiment showed that the decrease was not due to irreversible denaturation. Two-chain activator (obtained by limited plasmin cleavage) treated in the same manner showed only a 25% decrease in the amidolytic activity. Furthermore, the two-chain activator thus treated was still able to incorporate [^{3}H]DFP, while active-site labeling of the low molecular weight form of the B-chain from trypsin-treated t-PA could no longer be visualized (Figure 3). In t-PA digested with trypsin–Sepharose for 1 h, the B-chain exists in both the high and low M_r form. After this material had been exposed to denaturing gel filtration, only the high molecular weight form incorporated DFP (Figure 3).

The B_{1-24} peptide was purified by RP-HPLC and added to samples of native or degraded t-PA. The amidolytic activity of samples, previously depleted of the B_{1-24} peptide, was restored in a concentration-dependent manner upon addition of the purified peptide (Figure 4). Addition of the peptide to one-chain activator or trypsin-degraded t-PA where the B_{1-24} peptide had not been removed had no effect.

Cleavage of One-Chain t-PA with Endoproteinase Lys-C. After digestion of t-PA with endoproteinase Lys-C for 24 h, the major part of the protein had been converted to a two-chain form (Figure 5). Lysylprotease digested t-PA was reduced

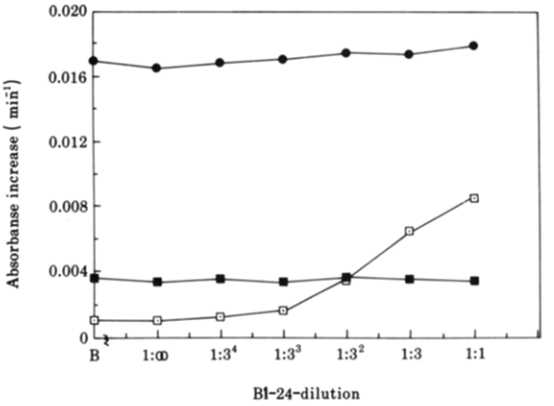

FIGURE 4: Addition of purified B_{1-24} peptide to t-PA and t-PA derivatives. The B_{1-24} peptide was isolated by RP-HPLC, lyophilized, and redissolved in 100 μL of 13 mmol/L of TFA. The concentration of the peptide was determined by amino acid analysis and using the known composition of the peptide. It was serially diluted with 0.1% TFA to give a concentration in the range from 7.5 μmol/L (0.15 μg/mL) to 0.09 μmol/L. Ten microliters of each dilution was added to 50 μL of (1) t-PA digested with trypsin for 4 h (●); (2) idem, but subjected to chromatography in 4 M guanidine hydrochloride (□); (3) native one-chain t-PA (■). The concentrations were in all three cases 10 μg/mL in 0.5 mol/L of ammonium bicarbonate, giving a final concentration of ~0.14–0.16 μmol/L. The addition of 10 μL of the diluted peptide (final concentration in the range 0.015–1.25 μmol/L) did not change the pH of the mixture. In the ∞ sample, 10 μL of 0.1% TFA was added, and in the B sample, 10 μL of ammonium bicarbonate was added. The mixtures were incubated at ambient temperature overnight, after which the samples were diluted 1:10 in NaCl/P_i and the amidolytic activity was determined as described under Material and Methods.

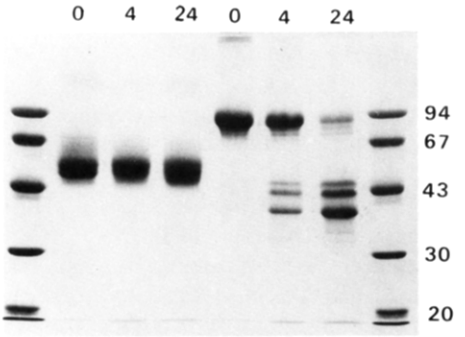

FIGURE 5: SDS–PAGE of lysylprotease-digested t-PA. Samples of lysylprotease-digested activator were reduced and carboxymethylated as described under Materials and Methods. The gel was stained with Coomassie Brilliant Blue. Positions of molecular weight markers and digestion times in hours are indicated. Nonreduced samples are shown in the left part of the figure and reduced samples in the right part.

and carboxymethylated, and the resulting peptides were separated by RP-HPLC. From the 24-h digest, a major peptide eluting at 44% acetonitrile was isolated. By SDS–PAGE, the molecular weight was found to correspond to that of the B-chain. The N-terminal amino acid sequence of this peptide started with G-G-L-F-A, showing a cleavage of the Lys_{280}–Gly_{281} bond two residues C-terminally of the trypsin or plasmin cleavage site. A peptide fraction containing the A-chain was also isolated. Its N-terminal sequence was S-Y-Q-I-, i.e., the N-terminus. A minor sequence starting with T-E-M-I-Y-(Thr_{14}) was also identified.

In contrast to trypsin, the lysylprotease-induced two-chain form had lower amidolytic activity than the starting material (Table IV). Furthermore, plasminogen activation in the absence of fibrin increased with only 30%, as compared to 250% after trypsin treatment. The amidolytic activity could not be further increased by subsequent plasmin–Sepharose proteolysis. Plasmin treatment had no effect on the lysyl-

Table IV: Enzymatic Activity of t-PA after Endoproteinase C Cleavage

time (h)	amidolytic activity in the absence of fibrin[a]	plasminogen activation in the absence of fibrin[a]	plasminogen activation in the presence of fibrin[a]
0	100	100	100
4	100	131	70
24	88	126	62

[a] Percent of starting value.

protease-induced two-chain activator. Kinetic analysis with a low molecular weight substrate (H-D-Ile-Pro-Arg-pNA) did not show any significant change in the kinetic parameters k_{cat} or K_m following the lysylprotease conversion to the two-chain form.

Discussion

The cleavage sites in proteolytically degraded t-PA have been determined by N-terminal amino acid sequence analysis of the fragments and correlated to changes in enzymatic properties observed at different times. As expected, a sensitive trypsin cleavage site was found at Arg_{278}–Ile_{279}, previously shown to be the plasmin cleavage site for conversion to two-chain t-PA (Wallén et al., 1983; Pohl et al., 1984). As observed with plasmin (Wallén et al., 1980; Rånby et al. 1982a), the hydrolysis of this bond induces a marked increase in the amidolytic activity (Table I).

Additional cleavages occurred in t-PA after treatment with trypsin–Sepharose. These were located in the A-chain and resulted in a marked degradation of this part of the activator. The finger domain seems to be particulary sensitive to proteolysis, and several cleavage sites were identified in this area. Preliminary data also indicate cleavages in the kringle 2 domain at the Arg_{252}–Arg_{253} bond, which may be the cause of the decreased affinity to arginine–Sepharose. Together the cleavages in the finger and kringle 2 domain could explain the rapid decrease in fibrin affinity and fibrin-stimulated plasminogen activation (Tables I and II).

An interesting cleavage occurred at the Arg_{302}–Ser_{303} bond, resulting in the generation of a 24 AA residue peptide (B_{1-24}). During the proteolytic activation of other serine proteases, the new N-terminus formed in the B-chain folds back and forms a salt bridge with an aspartate residue in the interior of the enzyme (Fersht, 1972; Blow, 1976; Huber & Bode, 1978). A similar mechanism could be expected in t-PA, since this area is highly homologous to other serine proteases (Strassburger et al., 1983). The amidolytic activity of t-PA was, however, unaffected by complete cleavage of the Arg_{302}–Ser_{303} bond (Table I), indicating a different mechanism or a high-affinity noncovalent interaction between the B_{1-24} peptide and the B-chain.

The high molecular weight fraction obtained by gel filtration of highly degraded t-PA under nondenaturing conditions retained high amidolytic activity (Table III) and contained the B_{1-24} peptide. In contrast, gel filtration in denaturing solvent (4 mol/L of guanidine hydrochloride) caused an almost complete loss of activity (Table III). RP-HPLC peptide mapping confirmed that the B_{1-24} peptide was removed.

Kinetic analysis with low molecular weight substrates of the t-PA derivative where the B_{1-24} peptide had been removed showed a marked decrease in the k_{cat} of the reaction. This could be due to a disruption of the charge-relay system in the active-site area. Together with the decreased K_m this indicates that substrate binding is still possible, but the catalytic activity is low.

The B_{1-24} peptide was easily identified in the RP-HPLC chromatogram and could be purified in small amounts. When added to a derivative of t-PA where the B_{1-24} peptide had previously been removed, the activity could be restored in a concentration-dependent manner (Figure 4). Addition of the B_{1-24} peptide to one- or two-chain activator, where the peptide had not been cleaved off or was still present, did not affect the amidolytic activity.

Treatment of single-chain t-PA with endoproteinase Lys-C results in a two-chain derivative. The B-chain of this form differs from the plasmin- or trypsin-derived B-chain in lacking the hydrophobic residue Ile_{279} and the positively charged Lys_{280} residue. In contrast to the plasmin-derived two-chain form, the cleavage at Lys_{280}–Gly_{281} did not induce any increase in the amidolytic activity. This indicates that the formation of a free N-terminal amino group is not the only prerequisite that must be fulfilled to generate an increased activity in two-chain t-PA.

In summary, the results from the trypsin and lysylprotease cleavages of t-PA demonstrate the important role of the N-terminal part of the B-chain for enzymatic activity of t-PA. These results also indicate that findings in experiment performed with t-PA derivatives, produced by site-directed mutagenesis, must be interpreted with some cautions as long as the complete three-dimensional structure of t-PA has not been determined by, e.g., X-ray crystallography.

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Purification and Characterization of Two Unique Forms of Cytochrome P-450 from Rabbit Nasal Microsomes†

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ABSTRACT: Two forms of cytochrome P-450, designated P-450NMa and P-450NMb, were purified to electrophoretic homogeneity from rabbit nasal microsomes. The purified cytochromes, which contained 14–16 nmol of P-450/mg of protein, exhibited apparent monomeric molecular weights of 49 500 and 51 000, respectively. As indicated by several criteria, including the amino acid composition, absorption spectra, and peptide maps, the two nasal forms of P-450 are distinct from each other. Furthermore, as judged by the NH_2-terminal amino acid sequences, they are distinct from all other P-450 cytochromes described to date. In the ferric form, P-450NMa is in the low-spin state, whereas P-450NMb is predominantly in the high-spin state. When reconstituted with NADPH–cytochrome P-450 reductase and phospholipid, P-450NMa is very active in the oxidation of ethanol as well as several nasal procarcinogens, including the N-deethylation of *N*-nitrosodiethylamine, the O-deethylation of phenacetin, and the N-demethylation of hexamethylphosphoramide. P-450NMb also metabolizes these substrates, but at lower rates. Both nasal forms are also active with testosterone, with P-450NMa oxidizing the substrate in the 17-position to give androstenedione and P-450NMb catalyzing hydroxylation in the 15α-, 16α-, and 19-positions. The two cytochromes represent the major portion of the total P-450 in nasal microsomes, but the corresponding forms could not be detected in hepatic microsomes.

The involvement of cytochrome P-450 in the oxidative metabolism of numerous xenobiotics as well as endogenous substances is well recognized. As reviewed recently (Black & Coon, 1986, 1987), more than 60 P-450s have been purified to electrophoretic homogeneity from various species, mostly from liver microsomes. The tissue-specific distribution of some of these isozymes has been established. For example, P-450 isozymes that are immunochemically indistinguishable from form 3a, the alcohol-inducible rabbit liver microsomal cytochrome P-450, have been identified in microsomal preparations of rabbit kidney and nasal mucosa, but not in other tissues, including adrenal, brain, heart, intestine, lung, ovary, spleen, testis, and uterus (Ding et al., 1986). Most P-450s that oxygenate xenobiotics have some distinguishing and some overlapping substrate specificities, and the genetically determined level of each of the expressed forms is at least partly responsible for the tissue-specific toxicity of many foreign compounds.

The mammalian olfactory tissue functions to detect odorants as well as pheromones (Gower et al., 1981). To fulfill these functions, it is constantly exposed to the external environment and is one of the major targets for the toxicity of many airborne xenobiotics. The specific content of P-450 in nasal microsomes is relatively high, being second only to that of hepatic microsomes among all rabbit tissues examined (Ding et al., 1986), and is higher in the olfactory mucosa than in the respiratory mucosa (Dahl et al., 1982). The P-450-dependent metabolism of a variety of substances in nasal mucosa (Ding et al., 1986; Brittebo, 1982, 1987; Hadley & Dahl, 1983; Dahl & Hadley, 1983; Brittebo et al., 1983; Löfberg & Tjälve, 1984; Brittebo & Rafter, 1984; Dahl & Brezinski, 1985; Reed et al., 1986; Longo et al., 1986) may be related to the ability of the organism to maintain olfactory sensitivity (Dahl et al., 1982) as well as to the susceptibility of this particular tissue to the toxic effects of many foreign compounds (Dahl & Hadley, 1983). A striking feature of the monooxygenase system of the nasal mucosa is that its catalytic activity, expressed as the turnover number for total P-450, is much higher than that of hepatic microsomes with many substrates, such as butanol (Ding et al., 1986), hexamethylphosphoramide (HMPA)[1] (Dahl & Brezinski, 1985), or ethoxycoumarin (Reed et al., 1986). The higher concentration of NADPH–cytochrome P-450 reductase in rabbit and rat nasal microsomes as compared to liver microsomes (Ding et al., 1986;

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[1] Abbreviations: HMPA, hexamethylphosphoramide; NDEA, *N*-nitrosodiethylamine; PA, phenacetin; PEG, poly(ethylene glycol); SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; P-450NM and -LM, nasal microsomal and liver microsomal cytochrome P-450, respectively; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; IgG, immunoglobulin G.

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