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Functional Role of Proteolytic Cleavage at Arginine-275 of Human Tissue Plasminogen Activator As Assessed by Site-Directed Mutagenesis

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ABSTRACT: Activation of the zymogen form of a serine protease is associated with a conformational change that follows proteolysis at a specific site. Tissue-type plasminogen activator (t-PA) is homologous to mammalian serine proteases and contains an apparent activation cleavage site at arginine-275. To clarify the functional consequences of cleavage at arginine-275 of t-PA, site-specific mutagenesis was performed to convert arginine-275 to a glutamic acid. The mutant enzyme (designated Arg-275 → Glu t-PA) could be converted to the two-chain form by *Staphylococcus aureus* V8 protease but not by plasmin. The one-chain form was 8 times less active against the tripeptide substrate H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide (S-2288), and the ability of the enzyme to activate plasminogen in the absence of fibrinogen was reduced 20-50 times compared to the two-chain form. In contrast, one-chain Arg-275 → Glu t-PA has equal activity to the two-chain form when assayed in the presence of physiological levels of fibrinogen and plasminogen. Fibrin bound significantly more of the one-chain form of t-PA than the two-chain form for both the wild-type and mutated enzymes. One- and two-chain forms of the wild-type and mutated plasminogen activators slowly formed complexes with plasma protease inhibitors, although the one-chain forms showed decreased complex formation with α_2-macroglobulin. The one-chain form of t-PA therefore is fully functional under physiologic conditions and has an increased fibrin binding compared to the two-chain form.

The serine proteases comprise a family of enzymes that function in a wide variety of physiological processes (Reich et al., 1975). These enzymes are initially synthesized as inactive precursors that have slightly different conformations than their active forms (Stroud et al., 1975). The mechanism of activation of these zymogens occurs through limited proteolysis at a specific site (Neurath, 1975). This cleavage (at position 15 of the chymotrypsin numbering system) permits the new, positively charged α-amino group of residue 16 to form an ion pair with the polypeptide backbone at Asp-194 (Matthews et al., 1967; Sigler et al., 1968). Correlated with the formation of this ion pair are conformational changes (Neurath et al., 1956; Freer et al., 1970) that have been reported to increase the overall reaction rates of trypsin and chymotrypsin by factors of up to 10^7 (Neurath, 1975).

Human tissue plasminogen activator (t-PA)[1] is a key component of the group of proteins involved with fibrin dissolution (Verstraete & Collen, 1986). The carboxy-terminal 252 amino acids of this 527 amino acid glycoprotein share considerable homology with other serine proteases (Pennica et al., 1983;

[1] Abbreviations: t-PA, tissue-type plasminogen activator; KIU, Kallikrein Inactivator Units; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

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Native amino acid sequence	Pro Gln Phe Arg Ile Lys Gly Gly
Native DNA sequence	G CCT CAG TTT CGC ATC AAA GGA G
Synthetic primer	G CCT CAG TTT GAA ATC AAA GGA G
Amino Acid sequence following mutagenesis	Pro Gln Phe Glu Ile Lys Gly Gly

FIGURE 1: Native sequence and synthetic primer utilized for site-directed mutagenesis. The amino acid and gene sequence of native t-PA is depicted in the first two lines. The synthetic primer utilized is depicted in the third line. The primer had a triplet that differed from the native gene at the underlined codon. The resulting amino acid sequence of the mutant enzyme (Arg-275 → Glu t-PA_{1c}) is presented in the last line.

Strassburger et al., 1983; Pohl et al., 1984; Vehar et al., 1984). The position analogous to the zymogen activation site of the other serine proteases is at arginine-275 in t-PA (Pennica et al., 1983). The significance of cleavage at this site in t-PA has been the subject of debate. As expected for a member of the serine protease family, it has been reported that the single chain form of this protease is considerably less active than the two-chain form and that any associated activity is due to contamination by activated t-PA (Andreasen et al., 1984; Ichinose et al., 1984). This is consistent with our understanding of the mechanism of activation of the serine proteases. Several groups, however, have reported considerable enzyme activity associated with the one-chain form (Rijken & Collen, 1981; Wallen et al., 1981; Rijken et al., 1982; Ranby, 1982; Ranby et al., 1982a,b). It has also been proposed that the two forms have equal activity in the presence of the physiological substrate (plasminogen) and cofactor (fibrin) (Rijken et al., 1982).

To circumvent cleavage of one-chain t-PA by itself or plasmin (Wallen et al., 1981, 1982; Nielsen et al., 1983; Andreasen et al., 1984), site-specific mutagenesis was performed to convert arginine at position 275 of t-PA to glutamic acid. The resulting mutant form is herein designated Arg-275 → Glu t-PA. One-chain and two-chain forms of the enzymes are indicated by t-PA_{1c} and t-PA_{2c}, respectively. In this paper we describe the results comparing wild-type plasminogen activator to the Arg-275 → Glu t-PA, which is incapable of being cleaved at the "activation" site.

Materials and Methods

Fibrinogen, Enzymobeads, and thrombin were from Calbiochem, Bio-Rad Laboratories, and Sigma Chemical Co., respectively. Plasmin and V8 protease were from Helena Laboratories and Miles Laboratories, respectively.

Enzymes. By use of in vitro mutagenesis (Adelman et al., 1983; Norris et al., 1983), the codon specifying arginine at position 275 of human t-PA was altered to encode a glutamic acid (Figure 1). The sequence of the resulting plasmid was confirmed by DNA sequence analysis. Recombinant t-PA was obtained from the supernatants of Chinese hamster ovary (CHO) cells, which were transfected with a plasmid capable of directing the expression of either the wild-type protein (Activase) or the Arg-275 → Glu t-PA (Pennica et al., 1983; Goeddel et al., 1983). t-PA and Arg-275 → Glu t-PA were purified, according to published procedures, on zinc chelate–Sepharose (Rijken & Collen, 1981) and lysine–Sepharose (Vehar et al., 1984). Glu–plasminogen was purified from human plasma by the procedure of Plow and Collen (1981) in the presence of aprotinin (10 KIU/mL). The aprotinin was removed by chromatography on a 2.5 × 90 cm Ultrogel resin in 0.02 M sodium phosphate, pH 7.5, containing 0.1 M sodium chloride. Plasminogen-containing fractions were dialyzed into 0.05 M Tris-HCl, pH 8.0, containing 0.1 M sodium chloride, aliquoted, lyophilized, and stored at −70 °C.

Protease–Sepharose Preparation. Cyanogen bromide activated Sepharose was prepared for coupling as described by the manufacturer (Pharmacia). Coupling was performed in 0.1 M sodium bicarbonate containing 0.5 M sodium chloride. Plasmin (800 μg) was incubated with 5 mL of activated resin for 16 h at room temperature. *Staphylococcus aureus* V8 protease (5 mg) was incubated with 5 mL of resin for 16 h at room temperature. Residual reactive groups on the resins were blocked by incubation with 1 M ethanolamine, pH 8.0, for 2 h at room temperature. To remove uncoupled protease, resins were washed extensively with 0.1 M ammonium bicarbonate, pH 8.0, containing 1.0 M sodium chloride, followed by washing with 0.1 M ammonium acetate, pH 4.0, containing 1.0 M sodium chloride.

Limited Proteolysis. The protease–Sepharose resins were each stored as a 50% slurry in 0.1 M ammonium bicarbonate, pH 8.0, containing 1.0 M sodium chloride. For limited proteolysis, 500 μL of the resin slurry was centrifuged at 10000*g* for 1 min in an Eppendorf centrifuge. The buffer was removed by aspiration, and 500 μL (100 μg) of either t-PA or Arg-275 → Glu t-PA was added. The reaction was allowed to proceed for 90 min at 35 °C while being rotated end over end to maintain the resin in suspension. Proteolysis was terminated by removal of the resin by centrifugation as described above.

Radiolabeling of t-PA and Arg-275 → Glu t-PA. Proteins (60–170 μg/mL; 200 μL per reaction) were radiolabeled by a 20-min incubation with Enzymobeads in the presence of 1 mCi of $Na^{125}I$. Free radiolabel was removed by chromatography through Sephadex G-25. The radiolabeling procedure yielded approximately 1×10^8 cpm/μg. It was observed that, following labeling, a significant amount of the radiolabeled protein, although functional, would no longer bind to lysine–Sepharose. The samples were therefore rechromatographed on lysine–Sepharose, and only the material that bound to the column was used in these studies.

Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on either 4–10% or 7–17% polyacrylamide gradient slab gels, using the buffer system of Laemmli (1970). Proteins were visualized according to the silver stain procedure of Morrissey (1981). The apparent molecular weights (M_r) of proteins observed upon SDS–PAGE analysis were calculated by extrapolation from the mobilities of protein standards (Bio-Rad).

In Vitro Plasma Studies. Freshly drawn, citrated human whole blood (500 μL) was incubated with 200000 cpm of ^{125}I-labeled enzyme at room temperature. In order to prevent generalized proteolysis by any plasmin that might be formed during the incubation, aprotinin was added to 200 μg/mL. Samples (50 μL) were removed after the indicated time and centrifuged to remove cells. Less than 5% of the label was associated with the cellular fraction. The samples were diluted with 10 volumes of 2% SDS sample buffer and analyzed on SDS–PAGE (500 cpm per lane). Complexes were detected by autoradiography.

Kinetic Analysis of S-2288 Cleavage. Kinetic constants for the hydrolysis of the tripeptide *p*-nitroanilide substrate H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide (S-2288) were obtained with an H-P diode array spectrophotometer (HP8451-A). Hydrolysis of substrate was measured continuously at two substrate concentrations (1.0 and 0.1 mM). The enzyme concentrations were as follows: Arg-275 → Glu t-PA_{1c}, 9.2×10^{-8} mol/L; Arg-275 → Glu t-PA_{2c}, 1.75×10^{-8} mol/L; t-PA_{2c}, 1.63×10^{-8} mol/L. Kinetic parameters were determined from a modified progress curve (Estell et al., 1985;

Table I: Kinetic Constants for Hydrolysis of S-2288

enzyme	K_m (mM)	k_{cat} (s^{-1})	K_{cat}/K_m (mM^{-1} s^{-1})
Arg-275 → Glu t-PA_{1c}	1.31 ± 0.06	7.93 ± 0.36	6.1
Arg-275 → Glu t-PA_{2c}	0.24 ● 0.01	10.5 ● 0.6	43.0
t-PA_{2c}	0.24 ● 0.01	8.32 ● 0.43	34.7

D. Estell, unpublished results).

Plasminogen Activation Assay. The reaction mixture contained 30 μL of a plasminogen solution (2.1 mg/mL) with and without fibrinogen (20 μL of a 20 mg/mL solution) in a final total volume of 150 μL of 0.05 M Tris-HCl, pH 7.4, 0.12 M sodium chloride, and 0.01% Tween 80. A total of 10 μL of the sample to be tested was added, containing 2–5 ng of enzyme for reactions with fibrinogen and between 0.05 and 2 μg for reactions without fibrinogen. The reaction was allowed to proceed for 10 min at 37 °C. The reaction mixture was diluted with 0.35 mL of 0.86 mM H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide (S-2251) dissolved in 36 mM Tris, 0.086 M NaCl, and 0.007% Tween 80. Plasmin cleavage of the S-2251 was allowed to proceed for 5 min, and the reaction was terminated by the addition of 25 μL of glacial acetic acid. The extent of plasmin formation was determined by the absorbance of the sample at 405 nm.

Fibrin Binding. A modification of the method of Rijken et al. (1982) was used. Fibrinogen (>98% clottable) was made plasminogen free by lysine–Sepharose chromatography (Deutsch & Mertz, 1970). The reaction mixtures contained 1 mg/mL human serum albumin, 500 ng/mL t-PA sample, and various amounts of fibrinogen in a buffer containing 0.05 M Tris-HCl, 0.12 M NaCl, and 0.01% Tween 80, pH 7.4. One unit per milliliter of thrombin was added to clot the mixtures. After 60 min at 37 °C, clots were removed from the solution by centrifugation and the supernatant was analyzed for free t-PA with a t-PA immunoassay.

RESULTS

Limited Proteolysis of t-PA_{1c} and Arg-275 → Glu t-PA_{1c}. Plasmin is known to cleave t-PA after arginine-275, resulting in a two-chain molecule linked by a disulfide bond (Wallen et al., 1981, 1982; Nielsen et al., 1983; Andreasen et al., 1984). It was anticipated that a similar limited proteolysis might occur upon exposure of Arg-275 → Glu t-PA_{1c} to V8 protease. The results of such proteolysis on both t-PA_{1c} and Arg-275 → Glu t-PA_{1c} are best evaluated by using SDS–PAGE under nonreducing and reducing conditions. SDS–PAGE analysis of t-PA_{1c} and Arg-275 → Glu t-PA_{1c} following exposure of each to plasmin and V8 protease is shown in Figure 2. Under nonreducing conditions, no effect on the mobility of either protein is observed, thereby demonstrating that exposure to either V8 or plasmin does not result in extensive degradation of the plasminogen activators and that any cleavage results in fragments that remain disulfide cross-linked.

Conversion of one-chain preparations to the two-chain form is easily detected by SDS–PAGE analysis of reduced preparations. Upon reduction, the two-chain material appears as a band with a M_r of approximately 30 000. Reduced SDS–PAGE analysis reveals a small amount of the t-PA_{2c} in the one-chain preparation (Figure 2). Exposure of t-PA_{1c} to plasmin–Sepharose resulted in the complete conversion to the two-chain form (t-PA_{2c}). That the cleavage was at arginine-275 was confirmed by amino acid sequence analysis (data not shown). As expected, exposure to V8–Sepharose had no effect on the wild-type molecule.

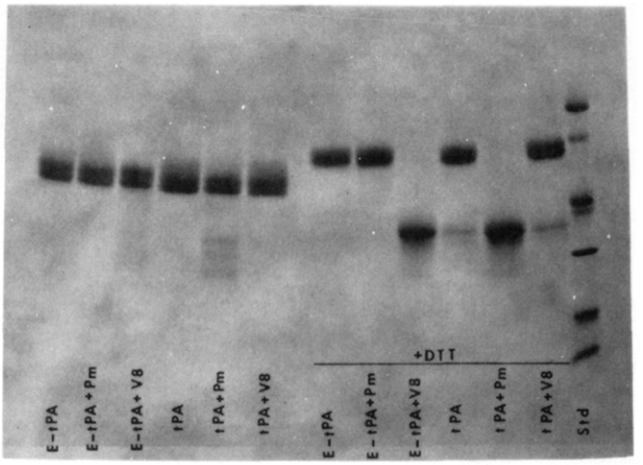

FIGURE 2: Activation of t-PA_{1c} and Arg-275 → Glu t-PA_{1c} by plasmin or V8 protease. Limited proteolysis was performed with either plasmin–Sepharose or V8 protease–Sepharose as described under Materials and Methods. Samples were analyzed on SDS–PAGE (7–17% polyacrylamide). Samples were reduced with DTT prior to electrophoresis as indicated. E–t-PA, Arg-275 → Glu t-PA_{1c}; E–t-PA + Pm, Arg-275 → Glu t-PA_{1c} incubated with plasmin–Sepharose; E–t-PA + V8, Arg-275 → Glu t-PA_{1c} incubated with V8 protease–Sepharose; t-PA, t-PA_{1c}; t-PA + Pm, t-PA_{1c} incubated with plasmin–Sepharose; t-PA + V8, t-PA_{1c} incubated with V8 protease–Sepharose; Std, molecular weight standards of 95 000, 68 000, 43 000, 31 000, 21 000, and 14 000.

Arg-275 → Glu t-PA_{1c} was resistant to plasmin–Sepharose treatment but was completely converted to the two-chain form (Arg-275 → Glu t-PA_{2c}) following exposure to V8–Sepharose (Figure 2). Amino acid sequence analysis confirmed selective cleavage at glutamic acid-275 (data not shown).

Kinetic Analysis of S-2288 Hydrolysis. Tripeptide *p*-nitroanilide substrates are useful in assessing the catalytic potential of a protease for small substrates. The ability of t-PA_{2c}, Arg-275 → Glu t-PA_{1c}, and Arg-275 → Glu t-PA_{2c} to hydrolyze the tripeptide substrate S-2288 is presented in Table I. Accurate kinetic analysis of t-PA_{1c} was not possible due to the contamination of all preparations with small amounts of t-PA_{2c}, which invalidated any calculated kinetic constants. Whereas the kinetic constants for Arg-275 → Glu t-PA_{2c} and t-PA_{2c} were found to be very similar, the kinetic constants for Arg-275 → Glu t-PA_{1c} were distinctly different (Table I). These results prove that the one-chain form of this mutant protease is less active than the two-chain form when the ability to hydrolyze small substrates is analyzed.

Plasminogen Activation in the Presence and Absence of Fibrinogen. The activation of plasminogen by the one-chain forms of the enzymes in the presence and absence of physiologic concentrations of fibrinogen is shown in Table II. The specific activity in the presence of fibrinogen was equivalent

Table II: Fibrinogen Stimulation of Plasminogen Activation[a]

	no treatment			plasmin–Sepharose			V8–Sepharose		
enzyme	−Fg	+Fg	stimulation (*x*-fold)	−Fg	+Fg	stimulation (*x*-fold)	−Fg	+Fg	stimulation (*x*-fold)
Arg-275 → Glu t-PA_{1c}	0.022	10.1	468	0.024	9.5	396	0.42	8.5	21.3
t-PA_{1c}	0.71	10.0	14.7	0.63	10.4	17.8	0.46	9.0	20.3

[a] Values are absorbance at 405 nm per milligram of plasminogen activator per minute.

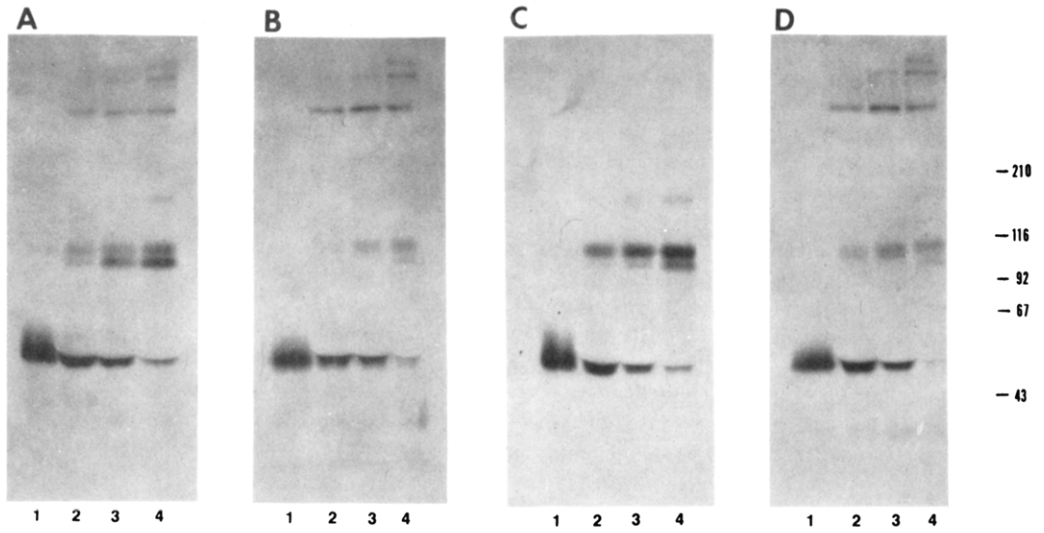

FIGURE 3: Plasma proteinase inhibitor complex formation of radiolabeled plasminogen activators. SDS–PAGE analysis of ^{125}I-labeled t-PA$_{1c}$ (A), t-PA$_{2c}$ (B), Arg-275 → Glu t-PA$_{1c}$ (C), and Arg-275 → Glu t-PA$_{2c}$ (D) following incubation in whole blood for 0 min (lane 1), 15 min (lane 2), 1 h (lane 3), and 16 h (lane 4). Plasma was obtained by centrifugation and analyzed on SDS–PAGE as described under Materials and Methods. The migrations of molecular weight standards are indicated at the right (in kilodaltons).

for all forms of the enzymes tested (+Fg columns). In contrast, untreated Arg-275 → Glu t-PA$_{1c}$ had 20–50 times less activity than t-PA$_{1c}$, t-PA$_{2c}$, or Arg-275 → Glu t-PA$_{2c}$ when assayed in the absence of fibrinogen. In this assay, therefore, the single chain form of the mutant protein has a lower ability to activate plasminogen in the absence of fibrinogen.

In Vitro Formation of Complexes with Proteinase Inhibitors of Human Plasma by Wild-Type and Mutant Enzymes. The time course of inactivation of the various radiolabeled forms of plasminogen activator by proteinase inhibitors in human plasma is shown in Figure 3. Both t-PA$_{1c}$ (Figure 3A) and t-PA$_{2c}$ (Figure 3B) slowly form SDS-stable complexes with proteinase inhibitors with calculated molecular weights (M_r) of 110 000 and 165 000, as well as a series of bands of M_r greater than 200 000. Arg-275 → Glu t-PA$_{1c}$ forms the complexes of M_r 110 000 and 165 000 but forms very little of the high molecular weight complexes (presumably α_2-macroglobulin) (Figure 3C). The rate and pattern of inhibitor complex formation of Arg-275 → Glu t-PA$_{2c}$ (Figure 3D) are almost identical with those of t-PA$_{2c}$ (Figure 3B).

Fibrin Binding. The ability of the various forms of plasminogen activator of the present study to bind to fibrin was assessed by incubation of the enzymes with increasing concentrations of fibrin, removal of the insoluble fibrin matrix, and determination of the amount of plasminogen activator removed form solution (Figure 4). t-PA$_{1c}$ was bound to fibrin to a greater extent than t-PA$_{2c}$. Arg-275 → Glu t-PA$_{1c}$ bound to an equivalent extent as t-PA$_{1c}$ and to a greater extent than Arg-275 → Glu t-PA$_{2c}$.

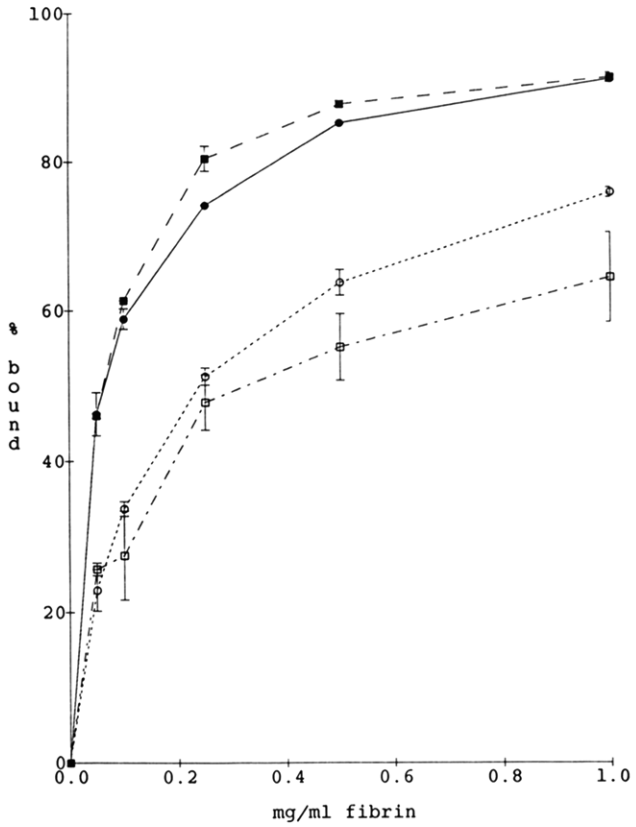

FIGURE 4: Binding of plasminogen activators to fibrin. Samples were incubated with different concentrations of fibrin and the percent bound was calculated by assaying the amount of the plasminogen activator remaining in the supernatant. Samples are as follows: (●) t-PA$_{1c}$; (■) Arg-275 → Glu t-PA$_{1c}$; (○) Arg-275 → Glu t-PA$_{2c}$; (□) t-PA$_{2c}$.

Discussion

This study documents the functional changes associated with the conversion of plasminogen activator from the one-chain form into the two-chain form. When the catalytic efficiencies of the various forms of t-PA and Arg-275 → Glu t-PA were assessed, it was determined that a less active zymogen state of t-PA$_{1c}$ does exist when catalytic activity is assessed by the hydrolysis of this tripeptide *p*-nitroanilide substrate. It must be pointed out, however, that the differences in activity are only 6–7 times, whereas for chymotrypsin and chymotrypsinogen the rate enhancements have been estimated at up to 7 orders of magnitude (Neurath, 1975). An exact determination of the k_{cat}/K_m for t-PA$_{1c}$ was not possible due to variable contamination by the two-chain form. It is assumed that a preparation composed solely of the single chain form would have values very similar to or identical with those of Arg-275 → Glu t-PA$_{1c}$.

A less active one-chain protein was also observed in assays of plasminogen activation in the absence of fibrinogen. Meaningful results from this type of assay would be extremely difficult to obtain with t-PA$_{1c}$ due to the plasmin-catalyzed generation of t-PA$_{2c}$ (Wallen et al., 1981, 1982; Nielsen et al., 1983; Andreasen et al., 1984). Arg-275 → Glu t-PA$_{1c}$, by

contrast, cannot be activated by plasmin (Figure 2) and so is the ideal molecule for the accurate determination of the catalytic capabilities of the one-chain form of the enzyme. Arg-275 → Glu t-PA_{1c} was found to be approximately 30 times less active than t-PA in such an assay (Table II, −Fg columns). In contrast, a markedly different ability of these proteins to activate plasminogen is seen when the assay is performed in the presence of fibrinogen. Although the proteolytic activity toward S-2288 depended upon the cleavage at position 275 (Table I), the ability of Arg-275 → Glu t-PA_{1c} to activate plasminogen in the presence of fibrinogen was found to be independent of cleavage at this site (Table II). Fibrinogen is apparently able to either cause a conformational change in the proteases and/or act as a cofactor, such that equal activities are observed in the one- and two-chain forms. Therefore, in the presence of physiological concentrations of fibrinogen and plasminogen, one-chain and two-chain molecules have equal activity and no zymogen state is observed.

Arg-275 → Glu t-PA_{1c} has a much greater fibrinogen-dependent stimulation of plasminogen activation than does t-PA_{1c} (Table II). This increase in stimulation, however, is not due to Arg-275 → Glu t-PA having a higher specific activity in the presence of fibrinogen than does t-PA but is due solely to a decreased activity of the Arg-275 → Glu t-PA_{1c} in the absence of fibrinogen.

In vitro experiments have indicated slow complex formation of human plasminogen activator plasma proteinase inhibitors (Korninger & Collen, 1981; Haggroth et al., 1984; Chmielewska et al., 1983; Juhan-Vague et al., 1984; Wiman et al., 1984a,b). The present study confirmed these observations. Notably, however, Arg-275 → Glu t-PA forms significantly less of the complexes of M_r greater than 200 000 (presumably α_2-macroglobulin complexes) (Figure 3C), indicating that the one-chain form of the protein does not react with this inhibitor. The complex formation observed in this region of the gel with t-PA (Figure 3A) is presumably dependent upon the conversion to t-PA_{2c} during the prolonged incubation in the plasma. The physiologic significance of these inhibitor complexes is unclear due to the slow rate of inhibitor complex formation compared to the rapid rate of clearance of uncomplexed plasminogen activator from the bloodstream (Collen, 1980; Korninger et al., 1981; Fuchs et al., 1985; Nielsen et al., 1983). Such complexes may, however, be of importance in the inactivation of plasminogen activator, which is bound to a fibrin clot and therefore exposed to proteinase inhibitors for longer periods of time.

The ability of plasminogen activator to bind to fibrin is shown in Figure 4. Contrary to a previous report (Rijken et al., 1982) where equal binding of t-PA_{1c} and t-PA_{2c} was observed, fibrin was found to bind significantly more t-PA_{1c} than t-PA_{2c}. Consistent with this observation, Arg-275 → Glu t-PA_{1c} and t-PA_{1c} bind equally well. Such control over fibrin binding may have physiological significance in the control of blood clot dissolution since plasminogen activator is synthesized and secreted in the one-chain form by vascular endothelial cells (Rijken et al., 1980) and converted to the two-chain form at the site of a clot by plasmin (Rijken et al., 1982) and by tissue kallikrein and factor Xa (Ichinose et al., 1984). The natural protein, therefore, is converted to a form with decreased fibrin affinity. Arg-275 → Glu t-PA, by contrast, cannot be cleaved to the two-chain form by known plasma proteases and will therefore remain in the form with higher fibrin binding properties. Work is in progress to generate a sufficient amount of the Arg-275 → Glu t-PA_{1c} in order to generate a detailed pharmacokinetic/pharmacodynamic profile in order to assess the in vivo fibrinolytic activity of Arg-275 → Glu t-PA compared to t-PA.

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Registry No. t-PA, 9001-91-6; S-2288, 77672-35-6; L-Arg, 74-79-3; L-Glu, 56-86-0; *Staphylococcus aureus* V8 protease, 66676-43-5; plasminogen, 9001-91-6; proteinase inhibitor, 37205-61-1.

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Articles

Predicted Structures of cAMP Binding Domains of Type I and II Regulatory Subunits of cAMP-Dependent Protein Kinase†

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ABSTRACT: The mammalian cAMP-dependent protein kinases have regulatory (R) subunits that show substantial homology in amino acid sequence with the catabolite gene activator protein (CAP), a cAMP-dependent gene regulatory protein from *Escherichia coli*. Each R subunit has two in-tandem cAMP binding domains, and the structure of each of these domains has been modeled by analogy with the crystal structure of CAP. Both the type I and II regulatory subunits have been considered, so that four cAMP binding domains have been modeled. The binding of cAMP in general is analogous in all the structures and has been correlated with previous results based on photolabeling and binding of cAMP analogues. The model predicts that the first cAMP binding domain correlates with the previously defined fast dissociation site, which preferentially binds N^6-substituted analogues of cAMP. The second domain corresponds to the slow dissociation site, which has a preference for C8-substituted analogues. The model also is consistent with cAMP binding in the syn conformation in both sites. Finally, this model has targeted specific regions that are likely to be involved in interdomain contacts. This includes contacts between the two cAMP binding domains as well as contacts with the amino-terminal region of the R subunit and with the catalytic subunit.

Cyclic AMP plays an important regulatory role in both prokaryotic and eukaryotic cells. The *Escherichia coli* catabolite gene activator protein (CAP) senses the level of cAMP and regulates transcription from several operons in the presence of cAMP (Zubay et al., 1970; Anderson et al., 1972). CAP binds to specific DNA sequences in the presence of cAMP and regulates transcription of several operons including lactose, galactose, and ara C [for review, see deCrombrugghe et al. (1984) and deCrombrugghe and Pastan (1978)]. The crystal structure of the CAP dimer with two bound molecules of cAMP has been determined (McKay & Steitz, 1981; McKay et al., 1982), and the amino acid sequence has been deduced from the DNA sequence of the gene (Aiba et al., 1982; Cossart & Gicquel-Sanzey, 1982). Each subunit of CAP folds into two domains; the larger amino-terminal domain binds cAMP between a long α-helix and a β-roll structure while the smaller carboxy-terminal domain forms the DNA binding site (Weber & Steitz, 1984; Steitz & Weber, 1985). The two subunits in the dimer have identical amino acid sequences but different orientations of the DNA binding domain with respect to the cAMP binding domain.

The major receptor for cAMP in eukaryotic cells is the regulatory subunit of cAMP-dependent protein kinase (EC 2.7.1.37) (Walsh et al., 1968). In the absence of cAMP, the kinase exists as an inactive tetramer containing two regulatory (R) and two catalytic (C) subunits. cAMP binds with high affinity to the regulatory subunit that promotes dissociation

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