Glycosylation at Asn-184 Inhibits the Conversion of Single-Chain to Two-Chain Tissue-Type Plasminogen Activator by Plasmin

Arthur J. Wittwer* and Susan C. Howard

Department of Cell Culture and Biochemistry, Central Research Laboratories, Monsanto Company, 800 North Lindbergh Boulevard, St. Louis, Missouri 63167

Received September 19, 1989; Revised Manuscript Received January 3, 1990

ABSTRACT: Tissue-type plasminogen activator (tPA) is a glycosylated serine protease which is an effective thrombolytic agent. Native single-chain tPA (sc-tPA) is converted to two-chain tPA (tc-tPA) by plasmin, the product of the reaction of plasminogen with tPA. Native sc-tPA occurs as two glycoforms. Type I sc-tPA is fully glycosylated, while type II lacks glycosylation at Asn-184. The rates at which type I and type II human melanoma sc-tPA were converted to type I and type II tc-tPA by plasmin were determined by two different methods. In each case, the second-order rate constant (k_{cat}/K_m) for type II sc-tPA (~8 μM^{-1} s^{-1}) was about twice that for type I sc-tPA (~4 μM^{-1} s^{-1}). These results indicate that glycosylation at Asn-184 hinders the conversion of sc-tPA to tc-tPA and suggest that under physiological conditions type I sc-tPA may persist in the single-chain form longer than type II sc-tPA. Previous studies have shown that type I tc-tPA has a lower activity than type II tc-tPA and that sc-tPA has a lower activity and susceptibility to inhibition when compared to tc-tPA. The present work provides further evidence that tPA glycosylation serves to modulate activity. The two major glycoforms may represent more persistant but slow acting (type I) and less persistent but faster acting (type II) variants of tPA.

Tissue-type plasminogen activator (tPA)[1] is a glycosylated serine protease of about 65K molecular weight which functions to activate the inactive zymogen plasminogen to the active fibrinolytic plasmin [see reviews by Bachmann and Kruithof (1984), Vehar et al. (1986), and Collen (1988)]. Endogenous tPA is responsible for the spontaneous lysis of blood clots (Wun & Capuano, 1985). Pharmacological doses of tPA have been shown effective in thrombolytic therapy [Van de Werf et al., 1984; Collen et al., 1984; and recent reviews by Braunwald (1988) and Tiefenbrunn and Sobel (1989)].

tPA is synthesized as a single-chain molecule which can be proteolytically cleaved to a two-chain form (Rijken & Collen, 1981). Plasmin catalyzes the cleavage of the Arg(275)–Ile bond in native single-chain tPA (sc-tPA) to form a disulfide bond linked two-chain species (tc-tPA) (Wallén et al., 1983). Since plasmin is the product of tPA's enzymatic activity, this conversion raises possibilities for a positive feedback type of regulation of tPA activity.

Two forms of tPA have been identified which differ in extent of glycosylation. Type I tPA is glycosylated at Asn-117, Asn-184, and Asn-448, while type II tPA is glycosylated only at Asn-117 and Asn-448 (Pohl et al., 1984). These two glycoforms can be separated by chromatography on lysine–Sepharose (Einarsson et al., 1985; Rijken et al., 1985). In previous work, both this and other laboratories have found that type II tPA has a significantly greater specific activity than type I tPA when measured in fibrin or fibrinogen fragment stimulated plasminogen activation assays (Einarsson et al., 1985; Wittwer et al., 1989). Our previous work compared the two-chain form of the enzymes. In the course of these studies, we noted that preparations of type I sc-tPA were more resistant to plasmin-catalyzed conversion to tc-tPA than were preparations of type II sc-tPA. Conversion of tPA from its single- to two-chain form is accompanied by a 5–7-fold increase in amidolytic activity toward artificial, chromogenic peptide substrates (Rånby et al., 1982b; Wallén et al., 1981). Thus, the increase in amidolytic activity of tPA can be used to monitor its conversion to the two-chain form. Using this method, it has been determined that the rate of conversion of sc-tPA to tc-tPA is about twice as fast for the less glycosylated type II glycoform than it is for the type I glycoform.

Materials and Methods

General. Human melanoma single-chain tissue-type plasminogen activator (sc-tPA), human plasmin, and reagents for enzyme-linked immunosorbant assay (ELISA) of tPA were obtained from American Diagnostica, Inc. Lysine–Sepharose and low molecular weight standards for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) were obtained from Pharmacia. The method of Laemmli (1970) was used for SDS–PAGE of reduced samples. Gels were stained with silver following the procedure of Morrissey (1981).

Lysine–Sepharose Chromatography. Types I and II sc-tPA were separated on lysine–Sepharose essentially as described (Parekh et al., 1989). A 1.6 × 96 cm column was equilibrated at 4 °C with 0.15 M KSCN, 120 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer, pH 8.0, containing 0.01% Tween 20 (Pierce) and 0.01% NaN_3. The t-PA sample in 1 M NH_4HCO_3 was then applied to the column and washed with at least 40 mL of equilibration buffer. The column was then developed with a linear gradient (of total volume 500 mL), beginning with equilibration buffer and ending with an elution buffer containing 0.6 M arginine hydrochloride, 0.25 M KSCN, 120 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer, pH 8.0, containing 0.01% Tween 20 and 0.01% NaN_3. Fractions (3 mL) were collected at 0.15 mL/min. The am-

* To whom correspondence should be addressed.

[1] Abbreviations: tPA, tissue-type plasminogen activator; sc-tPA, single-chain tPA; tc-tPA, two-chain tPA; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbant assay.

0006-2960/90/0429-4175$02.50/0

idolytic activity of these fractions was determined by using the chromogenic substrate S-2322 (H-D-Val-Gly-Arg-*p*-nitroanilide, KibiVitrum). The assay was performed in 96-well microtitration plates (Immulon 1, Dynatech). A 10-μL sample was added to a well followed by 90 μL of a reaction mixture composed of 1 part of 2.5 mg/mL S-2322, 1 part of 10× buffer [1 M NaCl, 1 mg/mL bovine serum albumin (BSA), 200 mM Tris-HCl, and 0.1% Tween 80, pH 7.6], and 7 parts of H_2O. The absorbance at 405 nm was monitored in a plate reader (Molecular Devices). Activities were calculated from the linear absorbance increase with time between the range 0 and 100 mAU, and were expressed as mAU per minute. On the basis of these activities, fractions were pooled for type I and type II. Each pool was concentrated to 1–2 mL using an Amicon pressure concentration cell with a YM-10 membrane and buffer exchanged by passing through a column of Sephadex G-25 (PD-10, Pharmacia) equilibrated with 1 M NH_4HCO_3. The tPA concentration in the resulting pools was determined by ELISA. Active-site titration with dansyl-Glu-Gly-Arg chloromethyl ketone (Calbiochem), using the indirect method of Magnotti (1988), indicated active-site concentrations which were 110% and 93%, respectively, of the protein concentrations determined by ELISA for the type I and type II glycoforms. This suggested that little or no differential inactivation or denaturation of the enzymes took place during isolation. In addition to similar active-site concentrations, it has previously been shown that the type I and type II species have equal direct amidolytic activity, whether protein is determined by amino acid analysis (Rånby et al., 1982b) or by ELISA (Wittwer et al., 1989). For these reasons, protein concentrations determined by ELISA were used to compare the type I and type II forms. Samples of tPA were stored at −70 °C until assay.

Assessment of sc-tPA Cleavage by Immobilized Antibody. An immobilized antibody having about 50-fold greater affinity for sc-tPA than for tc-tPA (PAM-1 Sepharose, American Diagnostica, Inc.) was used to directly assess the conversion of sc-tPA to tc-tPA by plasmin. In preliminary experiments, it was determined that this antibody bound the type I and type II forms of sc-tPA with similar affinity, and conditions were chosen to maximize the binding of sc-tPA without binding significant amounts of tc-tPA. Amidolytic assay reaction mixtures, as described above, containing 104 ng/mL type I or type II sc-tPA and 4 ng/mL plasmin were incubated at room temperature. At 10-min intervals, 50-μL portions were added to 50 μL of phosphate-buffered saline containing 0.01% Tween-80 and 0.01% sodium azide, 40 KIU/mL aprotinin, and about 15 μg of immobilized antibody (about 4 μL of resin). After several hours of occasional agitation, these mixtures were centrifuged, and the amount of tPA in the supernate was determined by a plasminogen-dependent indirect amidolytic assay (Wittwer et al., 1989). It was shown in initial work that sc-tPA and tc-tPA have equal activities in this assay and that the level of aprotinin was sufficient to stop any further tc-tPA formation without subsequently affecting the plasminogen-dependent activity assay. From these assay values, the percentage of tPA in the two-chain form at each time point was calculated by reference to control incubations lacking antibody. This control was required because of the significantly greater activity of type II compared to type I tPA in this assay (Wittwer et al., 1989).

Rate of sc-tPA Conversion: Discontinuous Method. The initial rate of conversion was determined at four tPA concentrations (19, 38, 75, and 151 ng/mL type I sc-tPA and 22, 44, 88, and 176 ng/mL type II sc-tPA) and a plasmin concentration of 5.9 ng/mL. The reaction mixture contained additionally 118 mM NaCl, 118 μg/mL BSA, 24 mM Tris-HCl buffer, pH 7.6, and 0.01% Tween 80. Samples of 85-μL volume were removed at 0.5, 1, 2, 4, and 8 min after initiation of the reaction by the addition of plasmin. These were added to wells of a microtitration plate containing 15 μL of a solution containing aprotinin and S-2322 such that the final concentrations were 1080 KIU/mL aprotinin and 0.25 mg/mL S-2322. At this concentration, aprotinin completely inhibited plasmin but did not affect the activity of tPA. The absorbance of these wells was then monitored at 405 nm and the amidolytic activity determined from the slope of an absorbance versus time plot, as described above for the analysis of lysine–Sepharose chromatography fractions.

Conversion to the two-chain form was evidenced by an increase in amidolytic activity with time. The slopes of plots of amidolytic activity versus time were determined by linear regression for each sc-tPA concentration. In this analysis, the 8-min time point was sometimes omitted to maintain linearity. These slopes, having units mAU/min per minute, were converted to nanograms per milliliter sc-tPA reacted (tc-tPA formed) per minute by reference to the amidolytic activity of control type I and type II sc-tPA measured under these same conditions both before and after exhaustive plasmin treatment to form tc-tPA. To do this, the slope values were divided by the difference between the amidolytic activities of tc-tPA and sc-tPA, expressed as mAU/min per ng/mL tPA. It was found that plots of initial sc-tPA conversion rate versus initial sc-tPA concentration were linear and passed through the origin, implying no departure from first-order kinetics in this range of substrate (sc-tPA) concentration. Assuming that the conversion of sc-tPA to tc-tPA by plasmin can be described by the Michaelis–Menten equation, $v = V_{max}S/(K_m + S)$, the observed linear relationship between reaction rate (v) and sc-tPA concentration (S) implied $S \ll K_m$ and $v = V_{max}S/K_m$. Hence, the apparent first-order rate constant, V_{max}/K_m, was calculated as the slope of the plots of v versus S by linear regression. These plasmin concentration dependent V_{max}/K_m values (in units of inverse time) were converted to plasmin concentration independent values (the second-order rate constant, k_{cat}/K_m, having units s^{-1} μM^{-1}) by dividing by the molar concentration of plasmin, calculated by using a molecular mass of 84 kDa, and dividing by 60 to convert min^{-1} to s^{-1}.

Rate of sc-tPA to tc-tPA Conversion: Continuous Method. (1) Theory. In a reaction mixture containing sc-tPA, plasmin, and S-2322, hydrolysis of S-2322 by tPA yields an absorbance increase at 405 nm, and conversion of sc-tPA to tc-tPA by plasmin will occur. The rate of absorbance increase, dA/dt, will be proportional to the concentrations of sc-tPA (S) and *tc*-tPA (P) and the respective pseudo-first-order rate constants, k_1 and k_2:

$$dA/dt = k_1S + k_2P \quad (1)$$

Equation 1 assumes that the S-2322 concentration does not change significantly over the course of the experiment and that the absorbance change is corrected for plasmin-mediated hydrolysis of S-2322. In practice, this correction was small (10% or less of the tPA-mediated hydrolysis), and measurements were restricted to a range (0–100 mAU) over which the concentration of S-2322 varied only by 6%. Recognizing that $S + P = S_0$, where S_0 is the initial concentration of sc-tPA, eq 1 can be written:

$$dA/dt = (k_1 - k_2)S + k_2S_0 \quad (2)$$

To obtain an expression for how S will decrease with time, it was found (see above) that at the concentrations of sc-tPA

used as substrate, the plasmin-mediated conversion to tc-tPA is essentially a first-order process; that is, these concentrations of tPA were significantly below the K_m. In this case, the concentration of sc-tPA is given by the expression:

$$S = S_0 \exp(-kt) \quad (3)$$

where k is V_{max}/K_m or $k_{cat}[\text{plasmin}]/K_m$ (Segel, 1968). Under these conditions, the amount of sc-tPA decreases exponentially, with a half-life of $\ln 2/k$.

Substituting eq 3 into eq 2 yields

$$dA/dt = (k_1 - k_2)S_0 \exp(-kt) + k_2S_0 \quad (4)$$

Integration of the above expression gives

$$A = (k_2 - k_1)S_0[\exp(-kt) - 1]/k + k_2S_0t + A_0 \quad (5)$$

where A is the absorbance at time t and A_0 is the initial absorbance at $t = 0$.

(2) *Experiment*. Absorbance measurements at 405 nm were made as a function of time for reaction mixtures containing the indicated levels of type I or type II sc-tPA and plasmin. Additionally, reaction mixtures contained 0.25 mg/mL S-2322, 100 mM NaCl, 100 μg/mL BSA, 20 mM Tris-HCl, pH 7.6, and 0.01% Tween 80. Reaction volumes of 100 μL were used in 96-well microtitration plates (Immulon 1, Dynatech) and were overlaid with 50 μL of mineral oil to prevent evaporation. Absorbance readings at 405 nm (650-nm reference) were obtained every 2 min in a Molecular Devices V_{max} plate reader.

Equation 5 was fit to the absorbance versus time data using multiple linear regression techniques. In this procedure, k_1 was determined directly in control experiments where plasmin was omitted from the reaction mixture. The remaining constants, k_2, k, and A_0, were then estimated by an iterative method described by Cleland (1967). Equation 5 can be rewritten

$$A - k_1S_0t = k_\Delta S_0\{[\exp(-kt) - 1]/k + t\} + A_0 \quad (6)$$

where $k_\Delta = k_2 - k_1$. If the nonlinear unknown constant k is estimated as k_0, then solution of the following linear model will yield a better estimate of k, symbolized as k':

$$A - k_1S_0t = k_\Delta S_0\{[\exp(-k_0t) - 1]/k_0 + t\} + (k' - k_0)k_\Delta S_0\{[1 - \exp(-k_0t)]/k_0^2 - t\exp(-k_0t)/k_0\} + A_0 \quad (7)$$

Equation 7 was solved for the unknown coefficients k_Δ, $(k' - k_0)k_\Delta$, and A_0 by using multiple linear regression. The better estimate of k derived from the above regression (k') was then used as k_0 in eq 7 for a second cycle of multiple linear regression. This process was then repeated until the determined coefficients did not change significantly. Plasmin concentration dependent values of k (V_{max}/K_m) were converted to k_{cat}/K_m values as described above for the discontinuous method.

Results

Figure 1 shows the separation of the type I and type II glycoforms of human melanoma sc-tPA on lysine–Sepharose. The less glycosylated type II glycoform has a higher affinity for the chromatographic resin and elutes at a higher concentration of arginine and KSCN than does type I. The profile shown in Figure 1 is very similar to that previously obtained for human melanoma tc-tPA, where it was rigorously demonstrated by tryptic peptide mapping that the two peaks represented the type I and type II glycoforms (Parekh et al., 1989). On this basis, the first peak (fractions 143–155) was identified as the fully glycosylated type I and the second (fractions 164–180) as type II, which lacks Asn-184 glycosylation. SDS–PAGE (Figure 1) verified the single-chain nature of the tPAs and the slightly larger molecular weight of type I compared to type II. Only traces of tc-tPA were evident in the preparations.

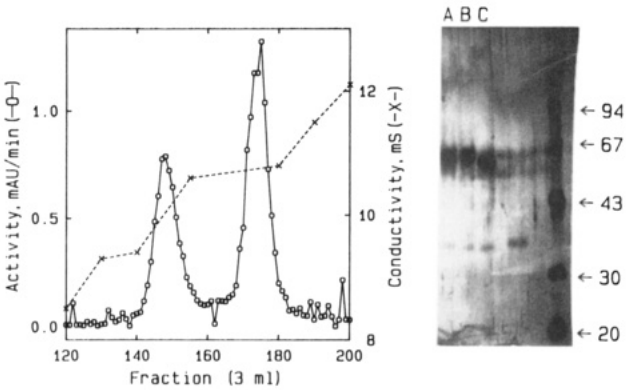

FIGURE 1: Separation of type I and type II sc-tPA on lysine–Sepharose. (Left panel) Chromatography of 0.44 mg of single-chain human melanoma tPA as described under Materials and Methods. Fractions 143–155 were pooled for type I, and fractions 164–180 were pooled for type II. Amidolytic acitivity in mAU per minute and conductivity in millisiemens are plotted for the indicated fractions. Only the relevant portion is shown. (Right panel) SDS–PAGE of unfractionated sc-tPA (lane A), type I sc-tPA (lane B), and type II sc-tPA (lane C). About 1 μg of each tPA was applied to the gel following reduction with 2-mercaptoethanol (see Materials and Methods). The migration positions of standard proteins are indicated: phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa). A sample of tc-tPA was completely dissociated under these conditions and migrated in the 33–37-kDa region (data not shown).

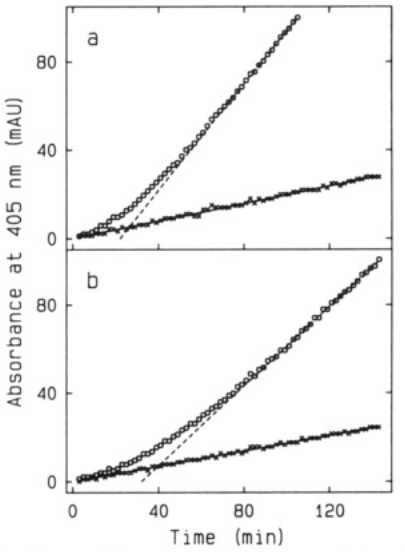

FIGURE 2: Absorbance of reaction mixtures containing the chromogenic tPA substrate S-2322 and (a) 75 ng/mL type II sc-tPA or (b) 64 ng/mL type I sc-tPA. For each panel, results are shown in the absence of plasmin (×) or in the presence of 5 ng/mL plasmin (○). See Materials and Methods for details. A solid line indicates the linear increase of absorbance with time for the reaction mixtures lacking plasmin as determined by linear regression. Dashed lines estimate the increase in reaction rate following plasmin treatment and were calculated by linear regression using data points in the range 60–100 mAU. Absorbances are corrected for blanks omitting tPA and including plasmin, where appropriate.

Hydrolysis of the chromogenic substrate S-2322 by either type I or type II sc-tPA resulted in a linear increase in absorbance with time, indicative of a constant amidolytic activity (Figure 2). The amidolytic activity of sc-tPA increases severalfold upon its conversion to tc-tPA by plasmin (Rånby et al., 1982b; Wallén et al., 1981). Indeed, if a catalytic

amount of plasmin was included in these reaction mixtures, the absorbance versus time plot curved upward, indicating the conversion of single-chain tPA to the more active two-chain species (Figure 2). The final apparent rate achieved when plasmin was present was 5–6 times that of sc-tPA alone. Similar results with the S-2322 amidolytic substrate have been reported by others (Rånby et al., 1982b).

The specific amidolytic activities calculated from the data presented in Figure 2 were essentially the same for the type I and type II tPAs before [2.7 and 2.6 mAU min^{-1} (μg/mL tPA)$^{-1}$, respectively] and after plasmin treatment [14.1 and 16.1 mAU min^{-1} (μg/mL tPA)$^{-1}$, respectively]. This agrees with previous findings that the amidolytic activities of these glycoforms are the same (Rånby et al., 1982b; Wittwer et al., 1989). Examination of Figure 2, however, suggested that the type II tPA was converted more rapidly to the more active, two-chain form. A similar plot of absorbance versus time for the unfractionated mixture of type I and type II sc-tPA suggested a rate of conversion intermediate between that seen for the isolated type I and type II species (data not shown).

Because of the low concentrations of tPA employed, it was impractical to use SDS–PAGE or reversed-phase HPLC of reduced samples to directly monitor sc-tPA cleavage. As an alternative method to verify that a physical cleavage of type I and type II sc-tPA was correlated with the activity changes seen upon plasmin treatment, an immobilized monoclonal antibody specific for sc-tPA was employed to separate sc-tPA from tc-tPA at various times after plasmin addition (see Materials and Methods). It was found that the percentage of total tPA not bound to the antibody increased with time, and this increase was more rapid for the type II preparation. For reaction mixtures initially containing 104 ng/mL type I or type II sc-tPA and 4 ng/mL plasmin, the type II incubation was found to be 47% tc-tPA after 30 min, compared with 24% tc-tPA for type I. After 60 min, these levels of tc-tPA increased to 65% and 36%, respectively. These results are consistent with what might be expected upon examination of Figure 2, where a somewhat higher concentration of plasmin and lower concentrations of tPA were used. By linear regression analysis of data in the 0–30-min range (data not shown), the average rate of cleavage of sc-tPA was 1.6% min^{-1} for type II sc-tPA and 0.9% min^{-1} for type I.

To better quantitate the more rapid cleavage by plasmin of type II compared to type I sc-tPA, the increase in amidolytic activity upon formation of the two-chain species was used to determine the kinetics of conversion for the two glycoforms. Two different approaches were employed: a discontinuous method based on initial rates and a continuous method based on a mathematical description of the absorbance versus time profiles shown in Figure 2.

In the discontinuous method (see Materials and Methods), the initial rate of conversion was determined at several sc-tPA concentrations by removing samples at various times. The reaction was stopped by the addition of aprotinin and the amidolytic activity measured. As expected, the amidolytic activity increased with time. Amidolytic activity was graphed as a function of time, and the slopes of these linear plots were proportional to the rates at which sc-tPA was converted to tc-tPA by plasmin. Conversion rates were about twice as fast for the less glycosylated type II tPA than for type I. These conversion rates were then plotted as a function of sc-tPA concentration for the type I and type II glycoforms. Over the concentration range employed (19–176 ng/mL), the relationship between conversion rate and tPA concentration was linear (data not shown). Assuming Michaelis–Menten kinetics, this suggested that these measurements were made significantly below the K_m of plasmin for type I and type II sc-tPA. This allowed the second-order rate constant, k_{cat}/K_m, to be determined from the slope of each linear plot. Values of 3.5 μM^{-1} s^{-1} for type I sc-tPA and 6.8 μM^{-1} s^{-1} for type II sc-tPA were obtained (Table I).

Table I: Rate of Conversion of Type I and Type II sc-tPA to tc-tPA As Determined by Two Methods[a]

tPA species	method[a]	plasmin concn (ng/mL)	tPA concn (ng/mL)	second-order rate constant, k_{cat}/K_m (μM^{-1} s^{-1})
type I	D	5.9	19–151	3.5
type I	C	5	64	4.6
type I	C	5	128	4.3
type I	C	10	128	3.7
type II	D	5.9	22–176	6.8
type II	C	5	75	8.6
type II	C	5	150	6.9
type II	C	10	150	7.7

[a] See Materials and Methods for details. Results are given for the discontinuous method (D), where the second-order rate constant was determined from the initial rates of sc-tPA conversion over the indicated range of tPA concentrations, and for a continuous method (C), where the second-order rate constant was determined directly by curve fitting.

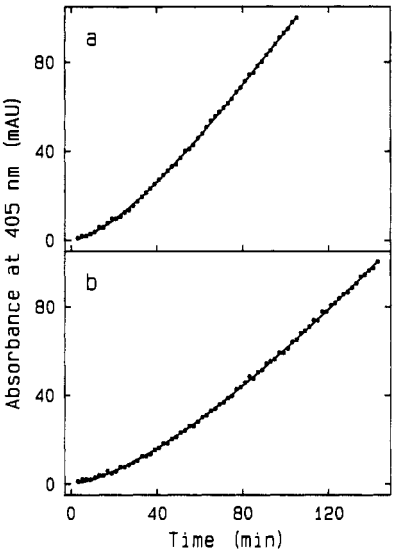

FIGURE 3: Absorbance versus time profiles for reaction mixtures containing the chromogenic tPA substrate S-2322, 5 ng/mL plasmin, and (a) 75 ng/mL type II sc-tPA or (b) 64 ng/mL type I sc-tPA. These data are identical with those given in Figure 2. The solid lines indicate the best fit to eq 6, from which the second-order rate constant was determined. See Materials and Methods for details.

The second-order rate constants for the conversion of type I and type II sc-tPA to tc-tPA were also determined directly by a continuous method in which the absorbance of a reaction mixture containing sc-tPA, plasmin, and S-2322 was monitored. Since the tPA concentrations employed appeared to be significantly below the K_m, the conversion could be described as a simple first-order process. An equation was derived, giving absorbance as a function of time, and this equation was fit to data such as those shown in Figure 2 (see Materials and Methods). Figure 3 indicates a good agreement between the experimental points of Figure 2 and the best-fit equation. Table I compares the second-order rate constants derived from these data and from two other sets of data obtained at other tPA or plasmin concentrations. These values are in good agreement with each other and with the second-order rate constants determined previously by the discontinuous, initial rate method. Both methods indicate an approximate 2-fold

greater conversion rate for type II sc-tPA compared to type I sc-tPA.

Discussion

The data presented here indicate that the plasmin-catalyzed cleavage of sc-tPA to the two-chain form occurs about twice as fast for type II tPA than for type I tPA. This suggests that glycosylation at site Asn-184, as occurs in type I but not type II tPA, inhibits the conversion of single- to two-chain tPA. Specific binding of the Asn-184 oligosaccharide to sites on plasmin or tPA may evoke conformational changes in the polypeptide, or the presence of a bulky oligosaccharide at Asn-184 may simply hinder the approach of the large (84 kDa) plasmin molecule to the Arg-275 cleavage site in tPA. Although the three-dimensional conformation of tPA in this region is not known, the oligosaccharide at Asn-184 is large enough to cover a considerable (3 × 2 nm) portion of the tPA surface (Parekh et al., 1989). It is therefore not inconceivable that interactions with plasmin or tPA could serve to slow the cleavage reaction.

Plasmin and plasminogen also exist in differently glycosylated forms, owing to the presence (form 1) or absence (form 2) of oligosaccharide at Asn-288 [see review by Castellino (1981)]. The influence of plasmin glycosylation state on the rate of sc-tPA to tc-tPA conversion was not studied in this report. However, acetic acid/urea gel electrophoresis (Suenson & Thorsen, 1981) showed the presence of both glycoforms in amounts previously seen by others (data not shown): about twice as much form 2 as form 1 (Castellino, 1981; Suenson & Thorsen, 1981; Nieuwenhuizen & Traas, 1989). Thus, the present study was performed using plasmin having a normal mixture of forms. Since form 1 plasminogen appears to be more easily activated to plasmin (Takada et al., 1985) and form 2 may be the predominant form bound to cellular receptors (Gonzalez-Gronow et al., 1989), it may be of physiological importance to also consider the effect of plasmin glycosylation on sc-tPA cleavage.

Petersen and Suenson (1986) determined the second-order rate constant for the conversion of unfractionated sc-tPA to tc-tPA by plasmin by monitoring changes in amidolytic activity with time. The value they obtained (1.0 μM^{-1} s^{-1}) is somewhat lower than the values for type I ($\sim$4 μM^{-1} s^{-1}) and type II ($\sim$8 μM^{-1} s^{-1}) sc-tPA obtained in this study. It is unlikely that the more rapid conversion seen in the present work is due to some effect of the isolation procedure on these species, since unfractionated sc-tPA appeared to have an intermediate conversion rate. Instead, it should be noted that reported values for the second-order rate constant for an analogous reaction, the plasmin-catalyzed conversion of single-chain urokinase-type plasminogen activator (sc-uPA) to its two-chain form, range from 0.05 to 3.3 μM^{-1} s^{-1} (Lijnen et al., 1986; Collen et al., 1986; Ellis et al., 1987; Peterson et al. 1988b). Recently, Scully et al. (1989) showed that a second-order rate constant of 3.5 μM^{-1} s^{-1} was obtained at low concentrations of sc-uPA (10–100 nM), while a much lower value (0.20 μM^{-1} s^{-1}) was obtained at higher concentrations (>0.3 μM). Thus, differences in the sc-tPA concentrations used in the present study (0.3–2.7 nM) and that of Peterson and Suenson (1986) (6.3 nM) may have been important. Limitations in the amount of sc-tPA fractionated during this study into type I and type II glycoforms have precluded measuring the second-order rate constant at higher concentrations of sc-tPA. It is of interest, however, that the second-order rate constant for plasmin-catalyzed sc-uPA cleavage measured at low sc-uPA concentrations is similar to that measured for type I sc-tPA in this study.

We have previously shown that type II tc-tPA has a catalytic rate constant for plasminogen activation which is about twice that of type I tc-tPA when determined in the presence of a fibrinogen fragment stimulator (Wittwer et al., 1989). The present report indicates that the single-chain forms of these tPA glycoforms differ by a similar magnitude in their efficiency as substrates for plasmin. The possible physiological consequences of this relate to the differing activities of sc-tPA and tc-tPA. Two-chain tPA reacts more rapidly than one-chain tPA with certain plasma protease inhibitors (Korninger & Collen, 1981; Rånby et al., 1982a) and with its rapid inhibitor PAI-1 (Chmielewska et al., 1988; Hekman & Loskutoff, 1988). Although there is general agreement that tc-tPA is a better plasminogen activator than sc-tPA in the absence of fibrin, most laboratories have concluded they have similar activity in the presence of fibrin or fibrinogen (Petersen et al., 1988a; Loscalzo, 1988; Tate et al., 1987; Rijken et al., 1982; Rånby, 1982) while others have found tc-tPA to be more active (Boose et al., 1989). If the greater susceptibility to inhibition and greater activity of tc-tPA are of physiological significance, then the prolonged existence of either endogenous or therapeutically administered type I sc-tPA in the single-chain form could both decrease its susceptibility to inhibition and delay any increase in fibrinolytic activity associated with conversion to the two-chain form. Combined with the decreased in vitro activity of type I compared with type II tPA, these factors are all consistent with the two glycoforms representing more persistent but slower acting (type I) and less persistent but faster acting (type II) variants of native tPA (Wittwer et al., 1989). In vivo testing will be required to substantiate this hypothesis and determine whether one glycoform presents a therapeutic advantage over the other.

The initial generation of plasmin during clot lysis by sc-tPA results in three potential plasmin-mediated positive feedback effects which could interact in regulating tPA activity. These include (1) conversion of N-terminal Glu-plasminogen to N-terminal Lys-plasminogen (Suenson & Thorsen, 1988), (2) degradation of fibrin to form more potent stimulatory species (Suenson et al., 1984; Norrman et al., 1985), and (3) conversion of sc-tPA to tc-tPA. The effect of glycosylation both on the single-chain to two-chain conversion rate and on fibrin-stimulated tPA activity may serve to modulate these feedback effects, allowing for enhanced biological control of the physiological fibrinolytic cascade.

Acknowledgments

We thank Linda S. Carr for expert technical assistance and Marilyn Reed for preparation of the manuscript.

References

Bachmann, F., & Kruithof, E. K. O. (1984) *Semin. Thromb. Hemostasis 10*, 6–17.

Boose, J. A., Kuismanen, E., Gerard, R., Sambrook, J., & Gething, M.-J. (1989) *Biochemistry 28*, 635–643.

Braunwald, E. (1988) *J. Am. Coll. Cardiol. 12*, 85A–92A.

Castellino, F. J. (1981) *Chem. Rev. 81*, 431–446.

Chmielewska, J., Rånby, M., & Wiman, B. (1988) *Biochem. J. 251*, 327–332.

Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 29*, 1–32.

Collen, D. (1988) *Thromb. Res., Suppl. VIII*, 3–14.

Collen, D., Topol, E. J., Tiefenbrunn, A. J., Gold, H. K., Weisfeldt, M. L., Sobel, B. E., Leinbach, R. C., Brinker, J. A., Ludbrook, P. A., Yasuda, I., Bulkley, B. H., Robison,

A. K., Hutter, A. M., Jr., Bell, W. R., Spadaro, J. J., Jr., Khaw, B. A., & Grossbard, E. B. (1984) *Circulation 70*, 1012–1017.
Collen, D., Zamarron, C., Lijnen, H. R., & Hoylaerts, M. (1986) *J. Biol. Chem. 261*, 1259–1266.
Einarsson, M., Brandt, J., & Kaplan, L. (1985) *Biochim. Biophys. Acta 830*, 1–10.
Ellis, V., Scully, M. F., & Kakkar, V. V. (1987) *J. Biol. Chem. 262*, 14998–15003.
Gonzalez-Gronow, M., Edelberg, J. M., & Pizzo, S. V. (1989) *Biochemistry 28*, 2374–2377.
Hekman, C. M., & Loskutoff, D. J. (1988) *Arch. Biochem. Biophys. 262*, 199–210.
Korninger, C., & Collen, D. (1981) *Thromb. Haemostasis 46*, 662–665.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lijnen, H. R., Van Hoef, B., & Collen, D. (1986) *Biochim. Biophys. Acta 884*, 402–408.
Loscalzo, J. (1988) *J. Clin. Invest. 82*, 1391–1397.
Magnotti, R. A., Jr. (1988) *Anal. Biochem. 170*, 228–237.
Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307–310.
Norrman, B., Wallén, P., & Rånby, M. (1985) *Eur. J. Biochem. 149*, 193–200.
Nieuwenhuizen, W., & Traas, D. W. (1989) *Thromb. Haemostasis 61*, 208–210.
Parekh, R. B., Dweck, R. A., Thomas, J. R., Opendakker, G., Rademacher, T. W., Wittwer, A. J., Howard, S. C., Nelson, R., Siegel, N. R., Jennings, M. G., Harakas, N. K., & Feder, J. (1989) *Biochemistry 28*, 7644–7662.
Petersen, L. C., & Suenson, E. (1986) *Biochim. Biophys. Acta 883*, 313–325.
Petersen, L. C., Johannessen, M., Foster, D., Kumar, A., & Mulvihill, E. (1988a) *Biochim. Biophys. Acta 952*, 245–254.
Petersen, L. C., Lund, L. R., Nielsen, L. S., Dano, K., & Skriver, L. (1988b) *J. Biol. Chem. 263*, 11189–11195.
Pohl, G., Kallstrom, M., Bergsdorf, N., Wallén, P., & Jörnvall, H. (1984) *Biochemistry 23*, 3701–3707.
Rånby, M. (1982) *Biochim. Biophys. Acta 704*, 461–469.
Rånby, M., Bergsdorf, N., & Nilsson, T. (1982a) *Thromb. Res. 27*, 175–183.
Rånby, M., Bergsdorf, N., Pohl, G., & Wallén, P. (1982b) *FEBS Lett. 146*, 289–292.
Rijken, D. C., & Collen, D. (1981) *J. Biol. Chem. 256*, 7035–7041.
Rijken, D. C., Hoylaerts, M., & Collen, D. (1982) *J. Biol. Chem. 257*, 2920–2925.
Rijken, D. C., Emeis, J. J., & Gerwig, G. J. (1985) *Thromb. Haemostasis, 54*, 788–791.
Scully, M. F., Ellis, V., Watahiki, Y., & Kakkar, V. V. (1989) *Arch. Biochem. Biophys. 268*, 438–446.
Segel, I. H. (1968) *Biochemical Calculations*, pp 390–395, Wiley, New York.
Suenson, E., & Thorsen, S. (1981) *Biochem. J. 197*, 619–628.
Suenson, E., & Thorsen, S. (1988) *Biochemistry 27*, 2435–2443.
Suenson, E., Lützen, O., & Thorsen, S. (1984) *Eur. J. Biochem. 140*, 513–522.
Takada, Y., Makino, Y., & Takada, A. (1985) *Thromb. Res. 39*, 289–295.
Tate, K. M., Higgins, D. L., Holmes, W. E., Winkler, M. E., Heyneker, H. L., & Vehar, G. A. (1987) *Biochemistry 26*, 338–343.
Tiefenbrunn, A. J., & Sobel, B. E. (1989) *Fibrinolysis 3*, 1–15.
Van de Werf, F., Ludbrook, P. A., Bergmann, S. R., Tiefenbrunn, A. J., Fox, K. A. A., Hilaire de Geest, C. B., Verstraete, M., Collen, D., & Sobel, B. E. (1984) *N. Engl. J. Med. 310*, 609–613.
Vehar, G. A., Spellman, M. W., Keyt, B. A., Ferguson, C. K., Keck, R. G., Chloupek, R. C., Harris, R., Bennett, W. F., Builder, S. E., & Hancock, W. S. (1986) *Cold Spring Harbor Symp. Quant. Biol. 51*, 551–562.
Wallén, P., Rånby, M., Bergsdorf, N., & Kok, P. (1981) *Prog. Fibrinolysis 5*, 16–23.
Wallén, P., Pohl, G., Bergsdorf, N., Rånby, M., Ny, T., & Jörnvall, H. (1983) *Eur. J. Biochem. 132*, 681–686.
Wittwer, A. J., Howard, S. C., Carr, L. S., Harakas, N. K., Feder, J., Parekh, R. B., Rudd, P. M., Dwek, R. A., & Rademacher, T. W. (1989) *Biochemistry 28*, 7662–7669.
Wun, T.-C., & Capuano, A. (1985) *J. Biol. Chem. 260*, 5061–5066.