Quenching of the Amidolytic Activity of One-Chain Tissue-Type Plasminogen Activator by Mutation of Lysine-416

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ABSTRACT: In contrast to most other serine proteases, tissue-type plasminogen activator (t-PA) possesses enzymatic activity as the one-chain zymogen form. The hypothesis that lysine residues 277 or 416 may be involved in stabilization of an active conformation of one-chain t-PA via salt-bridge formation with aspartic acid residue 477 was tested by site-directed mutagenesis. Four recombinant t-PA mutants were constructed. The amidolytic activities of these analogues were compared to that of authentic t-PA. Substitution of arginine-275 provided an analogue ([R275G]t-PA) resistant to plasmin cleavage. The amidolytic activity of [R275G]t-PA was comparable to that of authentic one-chain t-PA, and so was the activity of [R275L,K277L]t-PA, in which additional substitution of lysine residue 277 was carried out. This suggested that its presence was nonessential for obtaining one-chain t-PA activity. In contrast, substitution of lysine residue 416 to obtain [K416S]t-PA and [K416S,H417T]t-PA resulted in substantial quenching of amidolytic one-chain activity. As expected, the amidolytic activities of the two-chain forms were less affected by the substitution. Involvement of lysine residue 416 in one-chain t-PA activity was also indicated by decreased activities of [K416S]t-PA and [K416S,H417T]t-PA with plasminogen as the substrate. The one-chain activity of the lysine residue 416 substitution analogues was partially restored in the presence of fibrin. This could indicate that strong ligands such as fibrin might provide an alternative stabilization of the active conformation of one-chain t-PA.

Tissue-type plasminogen activator (t-PA)[1] belongs to the serine protease family. Generally, these proteins are synthesized and secreted by the cells as inactive one-chain zymogen forms. They become active enzymes only when subjected to limited proteolysis at a specific site. As exemplified by chymotrypsinogen, activation occurs as a result of proteolytic cleavage of an Arg_{15}–Ile_{16} peptide bond [see, e.g., Kraut (1977) and Pannekoek et al. (1988)]. The new Ile_{16} N-terminal then interacts with Asp_{194} adjacent to Ser_{195} of the catalytic Asp-His-Ser triad. Apparently such salt-bridge interaction is required to establish an active catalytic center. In analogy to this model, t-PA is assumed to be activated when the Arg_{275}–Ile_{276} peptide bond is split by plasmin (Wallén et al., 1983; Pennica et al., 1983) to generate two-chain t-PA. The N-terminal Ile_{276} is liberated to interact with Asp_{477} adjacent to Ser_{478}, which in homology to other serine proteases, together with His_{322} and Asp_{371}, is assumed to constitute the active triad of t-PA (Pennica et al., 1983; Pohl et al., 1984).

The activation of most serine proteases conforms to this general scheme, and the one-chain forms exist as genuine proenzymes; however, t-PA is an exception to this rule. It possesses significant activity in the one-chain form. Thus, the active state of one-chain t-PA has been illustrated by its ability to react with synthetic inhibitors (Wallén et al., 1982; Rånby et al., 1982; Smith, 1986; Green, 1986; Higgins & Lamb, 1986), with peptidyl nitroanilide substrates (Wallén et al., 1982; Rånby et al., 1982; Petersen & Suenson, 1986), and with the plasminogen activator inhibitors PAI-I and PAI-II (Kruithof et al., 1986; Åstedt et al., 1985). Recent studies with one-chain t-PA analogues resistant to plasmin cleavage obtained by recombinant techniques have now revealed that the specific activity of one-chain t-PA with its natural substrate plasminogen is about 3% of the activity of the two-chain form (Tate et al., 1987; Petersen et al., 1988; Boose et al., 1989). However, in the presence of fibrin, the activity of both one-chain and two-chain t-PA is dramatically enhanced to about the same high level of plasminogen activation (Tate et al., 1987; Petersen et al., 1988).

The mechanism by which an active conformation of one-chain t-PA is attained is not known. Wallén et al. (1983) have proposed that the one-chain t-PA activity might be due to an interaction between Asp_{477} and an appropriately positioned residue which could provide a positive charge for alternative salt-bridge formation. They pointed out that t-PA is unique among the serine proteases in having a lysine residue in position 2 (Lys_{277}) of the B-chain and suggested that its presence might explain the emzymatic activity of the one-chain form. The possible involvement of an alternative lysine residue (Lys_{416}) in salt-bridge formation has been suggested from a hypothetical three-dimensional model of the t-PA molecule (Heckel & Hasselbach, 1988). In the present work, we have tested the two possibilities by site-directed mutagenesis, and present experimental support for the involvement of lysine residue 416.

Materials and Methods

Reagents. The chromogenic substrates Val-Phe-Lys-*p*NA (S2390) (H-D-valyl-L-phenylalanyl-L-lysine-*p*-nitroanilide), <Glu-Gly-Arg-*p*NA (S2288) (L-pyroglutamyl-glycyl-L-arginine-*p*-nitroanilide), and Ile-Pro-Arg-*p*NA (S2288) (H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide) were obtained from Kabi (Stockholm, Sweden). Aprotinin was from Novo-Nor-

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[1] Abbreviations: t-PA, tissue-type plasminogen activator; Ile-Pro-Arg-*p*NA, H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide; <Glu-Gly-Arg-*p*NA, L-pyroglutamyl-glycyl-L-arginine-*p*-nitroanilide; Val-Phe-Lys-*p*NA, H-D-valyl-L-phenylalanyl-L-lysine-*p*-nitroanilide; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Lys_{78}-plasminogen, plasminogen (residues 78–791) with N-terminal lysine.

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disk (Bagsvaerd, Denmark). Human Lys_{78}-plasminogen (residues 78–791, but with minor amounts of residues 79–791) was prepared as described by Thorsen et al. (1981). Human fibrinogen (grade L) and human plasmin were from Kabi Stockholm, Sweden). Human thrombin was from Hoffmann-La Roche (Basel, Switzerland).

Recombinant DNA Techniques. The sequence of an authentic human t-PA cDNA clone has been reported (Pennica et al., 1983). In the present study, a cDNA clone comprising the coding sequence for mature t-PA was isolated by using mRNA from the Bowes melanoma cell line (Rijken & Collen, 1981) and t-PA-specific oligodeoxyribonucleotides. This cDNA was cloned in a plasmid designated pDR1296, and the *Escherichia coli* strain JM83 transformed with this plasmid has been deposited with the American Type Culture Collection under Assession No. 53347. Because the t-PA pre-pro sequence was not present in pDR1296, it was constructed from synthetic oligodeoxyribonucleotides and subsequently joined to the cDNA. In the synthesized t-PA pre-pro sequence, cleavage sites for *Bam*HI and *Eco*RI were introduced immediately 5′ to the initiating (ATG) codon while a *Nco*I site spanned the ATG codon. Likewise, the 3′ untranslated region of the t-PA cDNA was joined to a synthetic adaptor sequence with a *Bam*HI site. The synthetic 5′ (120 bp) and 3′ (40 bp) adaptors were annealed and then ligated to a 1.62-kb *Xho*II t-PA cDNA fragment from pDR1296 in a number of cloning steps to generate the plasmid ptPABam, in which full-length pre-pro tPA cDNA is cloned as a 1.78-kb *Bam*HI fragment in pUC13.

The t-PA cDNA was mutagenized (Kunkel et al., 1987) in an M13 vector to obtain the four mutants used in the present study. The cDNA sequences (coding strand) complementary to the oligonucleotides used for mutagenesis and the desired amino acid substitutions are

```
                                  Ser
[K416S]t-PA            CTCCGGCTACGGCTCCCATGAGGCCTTGTCT
                                  SerThr
[K416S,H417T]t-PA      CTCCGGCTACGGCAGTACTGAGGCCTTGTCT

                                Gly
[275G]t-PA               CCTCAGTTTGGCATCAAAGG
                                Leu   Leu
[R275L,K277L]t-PA         CTCAGTTTCTCATCCTCGG
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Synthesis of the oligonucleotides used for mutagenesis was performed on an Applied Biosystems Model 380-A DNA synthesizer.

Mutated t-PA cDNAs were cloned in the unique *Bam*HI site of the mammalian expression vector Zem219b to generate t-PA expression plasmids. Zem219b, which had previously been constructed (Eileen Mulvihill, ZymoGenetics, Seattle, WA) on the plasmid pUC18 has two mammalian gene units, which transcribe in different directions. (1) The mouse metallothionein promoter (MT) from pMThGH111 (Palmiter et al., 1983) directs the transcription of the different t-PA cDNAs; the human growth hormone gene terminator (HGH term.) from pEV142 (Low et al., 1985) is used as a terminator and polyadenylation signal. (2) The mouse dihydrofolate reductase (DHFR) cDNA from pDHFR-I (Berkner & Sharp, 1984) is expressed under control of the early promoter and terminator from SV40 (Buchman et al., 1981).

Expression of t-PA Mutants in Mammalian Cells. For expression of human t-PA mutants in cultured BHK cells [Syrian hamster kidney, thymidine kinase mutant line tk^-ts13 (Waechter & Baserga, 1982); American Type Culture Collection CRL 1632], the expression vectors were transfected into subconfluent cells by the calcium phosphate mediated transfection procedure (Graham & van der Eb, 1973). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). Forty-eight hours after transfection, cells were trypsinized and diluted into medium containing 400 nM methotrexate (MTX). After 10–12 days, individual colonies were isolated and assayed for t-PA expression with an ELISA assay (Brender & Selmer, 1983).

Purified t-PA was obtained from the cell culture supernatants as previously described (Selmer et al., 1983). Aprotinin (3 mg/L) was present during the purification procedure.

Conversion into the Two-Chain Form. This was performed by exposure of one-chain t-PA to 6 nM plasmin for 30 min followed by addition of 1 μM aprotinin (0.2 μM aprotinin in plasminogen activation experiments). Usually the conversion was performed in the reaction chamber just prior to the initiation of the reaction under investigation.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). This was performed on 10% polyacrylamide slab gels according to Laemmli (1970) followed by Coomassie blue staining. The apparent molecular weights (M_r) of proteins were calculated by extrapolation from the mobilities of protein standards.

Enzymatic Activity of t-PA. This was determined in buffer containing 50 mM Tris-HCl, 0.1 M NaCl, and 0.01% Tween 80, pH 7.4 (25 °C), using two different assays:

(*1*) *Direct Chromogenic Assay.* This assay employs the chromogenic peptidylanilide substrate Ile-Pro-Arg-*p*NA, which is cleaved directly by t-PA. The reaction mixtures (300 μL) were added to the wells of a 96-well microtiter plate, and reading of OD_{405nm} was performed by a Micro ELISA Autoreader MR 580 from Dynatech Laboratories.

(*2*) *Indirect Chromogenic Assay.* This was performed as previously described (Petersen et al., 1985). In this assay, a chromogenic substrate for plasmin was used to monitor the rate of plasminogen activation. The reaction mixture contained plasminogen activator, plasminogen, aprotinin, and Val-Phe-Lys-*p*NA. After a short pre-steady-state period, the generation of plasmin was exactly balanced by its inactivation by aprotinin. With a constant rate of plasminogen activation, this resulted in a constant steady-state level of plasmin, and with chromogenic plasmin substrate in the reaction mixture, this was observed as a constant rate of nitroaniline production (d[pNA]/dt). The rate of plasminogen activation (v_a) is related to d[pNA]/dt by

$$v_a = \frac{k_i K_s}{k_e}\frac{[I]}{[S]}\frac{d[pNA]}{dt} = (8.4 \times 10^{-2})\frac{[I]}{[S]}\frac{d[pNA]}{dt} \quad (1)$$

where $k_i = 5.6 \times 10^4$ $M^{-1}\cdot s^{-1}$ (Petersen & Clemmensen, 1981) is the rate constant for plasmin inactivation by aprotinin and where $k_e = 20$ s^{-1}, $K_s = 3 \times 10^{-5}$ M are the k_{cat} and K_m for plasmin cleavage of Val-Phe-Lys-*p*NA (values according to the manufacturer). Equation 1 suggests that for a given v_a, the same d[pNA]/dt is obtained at different concentrations of aprotinin [I] and chromogenic substrate [S], provided the ratio [I]/[S] is kept constant.

Fibrin-enhanced plasminogen activation was obtained with fibrinogen present in the reaction mixture to which t-PA and, immediately after, thrombin were added to start the reaction (Petersen et al., 1988). Fibrin formation took place within 5 min. At the low concentration of fibrinogen used, polymerization did not interfere significantly with the OD_{405nm} measurements.

Results

Identification of Potential Partners for Salt-Bridge Interaction with Asp_{477} in One-Chain t-PA. A search for possible candidates for salt-bridge formation with Asp_{477} in one-chain

Table I: Alignment of the Amino Acid Sequences of t-PA, Chymotrypsinogen, Trypsin, Elastase, and Kallikrein[a]

```
         277      I                                      II    322
           ↓                                                     *
t-PA   · |IKGGLFADIASHPWQAAI| FAKHRRSPGERF |LCGGILISSCWILSAAHC| FQERFPPHHLTVILGRTYRVVPGEEEQKFE
            20        30               40         50         60             70        80
Chym   · |IVNGEEAVPGSWPWQVSL| QDK      TGFH |FCGGSLINENWVVTAAHC| GVT     TSDVVVAGEFDQGSSSEKIQKLK
Tryp   · |IVGGYTCGANTVPYQVSL| NS        GYH |FCGGSLINSQWVVSAAHC| YKS      GIQVRLGQDNINVVEGNEQFIS
Elast  · |VVGGTEAQRNSWPSQISL| QYRSGS   SWAH |TCGGTLIRQNWVMTAAHC| VDRE    LTFRVVVGEHNLNQNNGTEQYVG
Kalli  · |IIGGRECEKNSHPWQVAI| YK       YSSH |QCGGVLVNPKWVLTAAHC| KND      NYEVWLGRHNLFENENTAQFFG

                                   371        III                                IV 416
                                    *                                                ↓
t-PA   · VEKYIVHKEFDDDTYD          |NDIALLQLKSDSSRCAQESSVVRTVCLP| PADLQLPDWTECE |LSGYGKH| E   ALSPFYSER
               90        100                     110        120          130      140             150
Chym   · IAKVFKNSKYNSLTIN          |NDITLLKLS     TAASFSQTVSAVCLP| SASDDFAAGTTCV |TTGWGLT| R   YTNANTPDR
Tryp   · ASKSIVHPSYNSNTLN          |NDIMLIKLK     SAASLNSRVASISLP| T  SCASAGTQCL |ISGWGNT| K   SSGTSYPDV
Elast  · VQKIVVHPYWNTDDVAAG        |YDIALLRLA     QSVTLNSYVQLGVLP| RAGTILANNSPCY |ITGWGLT| R    TNGQLAQT
Kalli  · VTADFPHPGFNLSADGKDYS      |HDLMLLRLQ     SPAKITDAVKVLELP| T  QEPELGSTCE |ASGWGSI| EPGPDDFEFPDE

         429            V                                    VI 478
          ↓                                                       *
t-PA   · |LKEAHVRLYPSSRCTSQHLLNRTVTDNMLCAG| DTRSGGPQANLH |DACQGDSGGPLV| CLNDGR
              160        170          180                 190      200
Chym   · |LQQASLPLLSNTNCKK  YWGTKIKDAMICAG|  ASGV        |SSCMGDSGGPLV| CKKNGA
Tryp   · |LKCLKAPILSNSSCKS  AYPGQITSNMFCAG| YLEGGK       |DSCQGDSGGPVV| CS  G
Elast  · |LQQAYLPTVDYAICSSSSYWGSTVKNSMVCAG|  GNRGV       |SGCQGDSGGPLH| CLVNGQ
Kalli  · |IQCVQLTLLQNTFCAD  AHPDKVTESMLCAG| YLPGGK       |DTCMGDSGGPLI| CN  G

                         VII
t-PA   · MTL |VGIISWGL  GCGQKDVPGVYTKVTNYLDWIR| DNMRP
              210       220         230          240
Chym   · WTL |VGIVSWGS STCS TSTPGVYARVTALVNWVQ| QTLAAN
Tryp   ·  KL |QGIVSWGS  GCAQKNKPGVYTKVCNYVSWIK| QTIASN
Elast  · YAV |HGVTSFVSRLGCNVTRKPTVFTRVSAYISWIN| NVIASN
Kalli  ·  MW |QGITSWGH TPCGSANKPSIYTKLIFYLDWID| DTITENP
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[a] Alignment using one-letter symbols and chymotrypsinogen numbering. Boxes (I–VII) designate structurally conserved regions (Greer, 1981). Dotted areas represent amino acids in which the α-carbons are within 8 Å from the γ-carboxy group of Asp_{194} in the X-ray crystal structure. Top-line numbering, specific t-PA amino acid residues mentioned in the text. Asterisks indicate catalytic residues.

t-PA was performed by inspection of known crystal structures of chymotrypsinogen, trypsin, elastase, and kallikrein with coordinate data extracted from the Protein Data Bank (Bernstein et al., 1977). The interaction was assumed to involve a positively charged residue, the α-carbon atom of which was within 8 Å from the γ-carbon of the aspartic acid residue equivalent to Asp_{194} of chymotrypsinogen. The results is shown in Table I, where the serine proteases are aligned (chymotrypsinogen numbering) as described by Furie et al. (1982) and Strassburger et al. (1983). Residues positioned within 8 Å from the γ-carbon of this aspartic acid are indicated by the shaded areas. If it is assumed that an equivalent spheric section of the t-PA serine protease domain is within the range of Asp_{477}, this identifies two lysine residues (Lys_{277} and Lys_{416}) as potential candidates for salt-bridge formation in one-chain t-PA. Lys_{429} was also identified by this procedure but only in chymotrypsinogen. This and also the fact that trypsinogen possesses a lysine residue in that position without having significant intrinsic activity led us to assume that salt-bridge interaction with Lys_{429} was less likely than with the other two lysine residues. His_{417} is also potentially close to Asp_{477}. Its presence might be important to the salt-bridge interaction of Lys_{416}, or it might otherwise indirectly interfere with salt-bridge formation.

Construction and Expression of One-Chain t-PA Analogues. The amino acid residues Arg_{275}, Lys_{277}, Lys_{416}, and His_{417} were replaced by other amino acids by oligonucleotide-directed mutagenesis of the t-PA cDNA as described under Materials and Methods. Successful mutagenesis was confirmed by DNA sequence analysis, and four different mutant cDNAs were expressed in transformed BHK cell lines. The t-PA mutants designated [R275G]t-PA, [R275L,K277L]t-PA, [K416S]t-PA, and [K416S,H417T]t-PA (with amino acid substitutions indicated by the one-letter code in parentheses) were purified from culture media. Reduced SDS–PAGE of four t-PA analogues is shown in Figure 1. A predominant band of the one-chain form (M_r 66 000) was observed in each case. Only trace amounts of two-chain t-PA (M_r 30 000–35 000) could be detected.

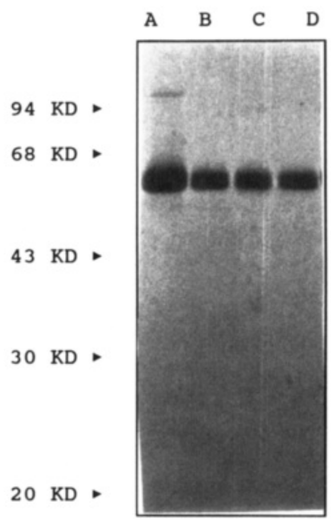

FIGURE 1: Reduced SDS–PAGE of t-PA analogues. Lane A, 16.4 μg of [K416S,H417T]t-PA; lane B, 7.8 μg of [K416S]t-PA; lane C, 8.2 μg of [R275G]t-PA; lane D, 9.7 μg of [R275L,K277L]t-PA.

Effect of Lys_{277} Substitution on Amidolytic Activity. Figure 2 compares the amidolytic activity of the one-chain analogues

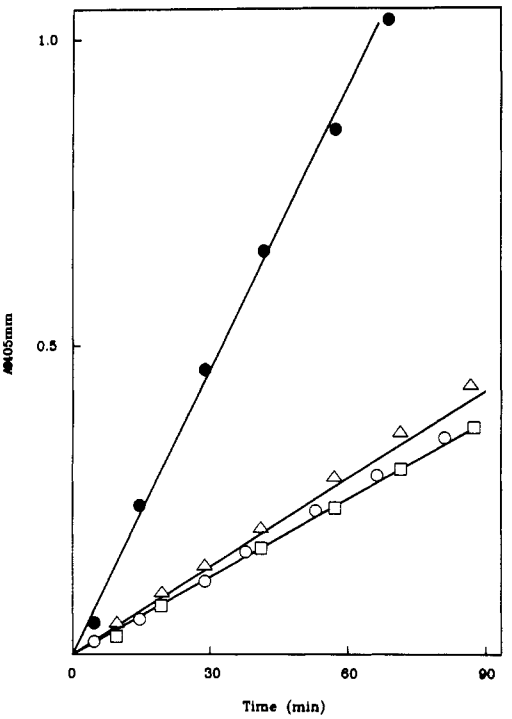

FIGURE 2: Effect of amino acid substitution on amidolytic activity. Substitution of Lys_{277}. Progress curves for the generation of *p*NA measured from OD_{405nm}. Comparison of the amidolytic activity of [R275G]t-PA (△) and [R275L,K277L]t-PA (□) with that of the one-chain form (○) and two-chain form (●) of authentic t-PA. The reaction mixture contained 10 nM t-PA, 0.3 mM Ile-Pro-Arg-*p*NA, 1.0 μM aprotinin, 0.05 M Tris-HCl, 0.1 M NaCl, and 0.01% Tween 80, pH 7.4.

Table II: Amidolytic Activity of [K416L]t-PA[a]

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
one chain			
authentic	0.8	2.9	3.6×10^3
[K416L]t-PA	2.0	0.54	0.3×10^3
two chain			
authentic	0.4	6.7	17×10^3
[K416L]t-PA	0.2	3.4	17×10^3

[a] Conditions: 50 mM Tris-HCl, 0.1 M NaCl, 0.01% Tween 80, pH 7.4 (25 °C).

[R275G]t-PA and [R275L,K277L]t-PA to the activity of one- and two-chain native t-PA. Substitution of Arg_{275} provides an analogue resistant to plasmin cleavage with an intact one-chain activity (Tate et al., 1987; Petersen et al., 1988; Boose et al., 1989). Figure 2 confirms this observation. The activity of [R275G]t-PA with the substrate Ile-Pro-Arg-*p*NA was identical with that of one-chain t-PA. Additional substitution of Lys_{277} to obtain [R275L,K277L]t-PA did not change the specific amidolytic activity significantly (Figure 2).

Effect of Lys_{416} Substitution on Amidolytic Activity. Figure 3 (open symbols) shows the activity on one-chain [K416S]t-PA and [K416S,H417T]t-PA as compared to authentic one-chain t-PA. The activity of both substitution analogues was clearly much lower than that of authentic t-PA. This strong quenching of amidolytic activity was not observed when the one-chain forms were converted into their two-chain counterparts (closed symbols). The amidolytic activity of two-chain t-PA appeared to be only moderately quenched by substitution of Lys_{416}.

The change in catalytic properties induced by the substitution of Lys_{416} is further characterized in Table II, which lists the kinetic constants obtained from Linewater–Burk plots of

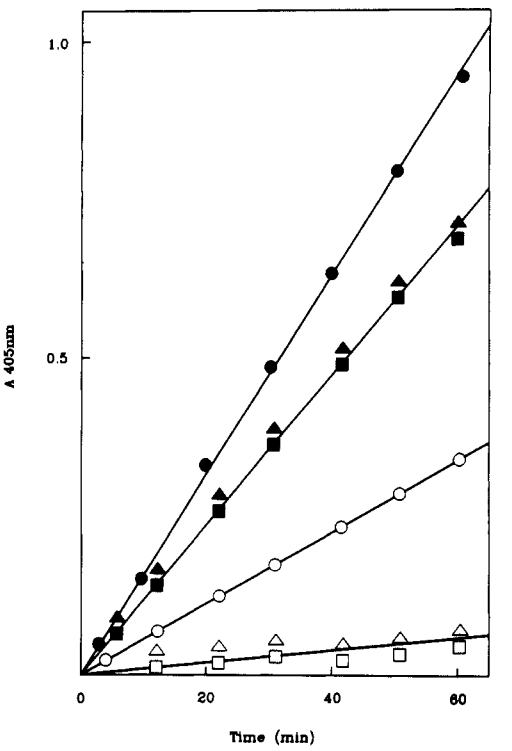

FIGURE 3: Effect of amino acid substitution on amidolytic activity. Substitution of Lys_{416}. Progress curves for the generation of *p*NA measured from OD_{405nm}. Comparison of the amidolytic activity of [K416S]t-PA (△, ▲) and [K416S,H417T]t-PA (□, ■) with that of authentic t-PA (○, ●). Open symbols, one-chain forms. Closed symbols, two-chain forms. Other conditions were as described in Figure 2.

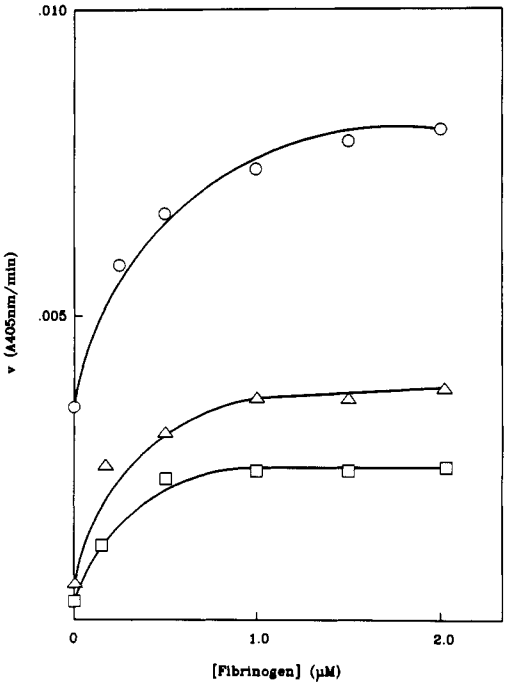

FIGURE 4: Effect of Lys_{416} substitution on fibrinogen-enhanced amidolytic activity. The activity of 10 nM one-chain authentic t-PA (○), [K416S]t-PA (△), and [K416S,H417T]t-PA (□) with Ile-Pro-Arg-*p*NA was measured in the presence of various concentrations of fibrinogen. Other conditions were as described in Figure 2.

rate as a function the of Ile-Pro-Arg-*p*NA concentration.

Effect of Lys_{416} Substitution on Fibrinogen-Enhanced Amidolytic Activity. The amidolytic activity of one-chain t-PA, but not of two-chain t-PA, is enhanced by fibrin(ogen) binding (Rånby et al., 1983). Figure 4 shows the effect of

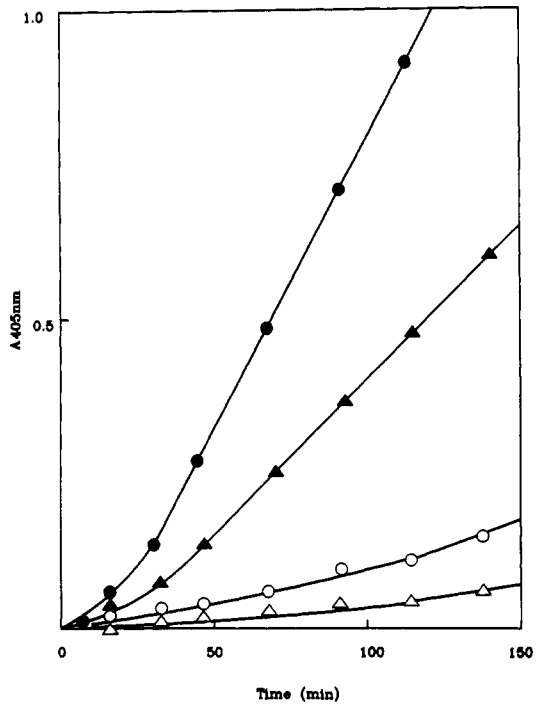

FIGURE 5: Effect of Lys_{416} substitution on plasminogen activation in the absence of fibrin. The plasminogen activation activity with [K416S]t-PA (△, ▲) and authentic t-PA (○, ●) was measured by the indirect chromogenic assay. Open symbols, one-chain t-PA. Closed symbols, two-chain t-PA. Additions: 10 nM t-PA, 0.3 μM Lys_{78}-plasminogen, 0.2 μM aprotinin, and 0.6 mM Val-Phe-Lys-*p*NA.

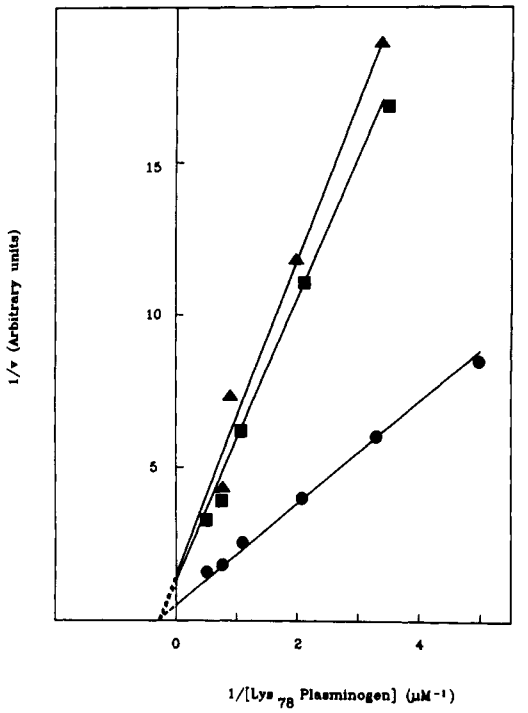

FIGURE 6: Effect of Lys_{416} substitution on two-chain t-PA activity. Double-reciprocal plot of plasminogen activation rate against Lys_{78}-plasminogen concentrations. The rate was measured from progress curves such as those shown in Figure 4 (closed symbols). The plasminogen activation kinetics of the two-chain form of [K416S]t-PA (▲) and [K416S,H417T]t-PA (■) were compared to that of authentic two-chain t-PA (●). Additions: 10 nM t-PA, 0.2 μM aprotinin, 0.6 mM Val-Phe-Lys-*p*NA, and various fixed concentrations of Lys_{78} plasminogen.

fibrinogen on the amidolytic activity of one-chain t-PA with Ile-Pro-Arg-*p*NA. In accordance with previous findings (Rånby et al., 1983; Tate et al., 1987; Urano et al., 1989), fibrinogen induces a marked enhancement of the activity of native one-chain t-PA. Fibrinogen-induced enhancement was also observed with the substitution analogues [K416S]t-PA and [K416S,H417T]t-PA (Figure 4). The low activity of these analogues with Ile-Pro-Arg-*p*NA was markedly stimulated, indicating that the mechanism by which fibrinogen enhances the activity does not require the presence of the Lys_{416} residue. Similar results were obtained for one-chain t-PA activity with the substrate <Glu-Gly-Arg-*p*NA (S2444). The activity was enhanced by fibrinogen when this was added as described in Figure 4. Furthermore, the application of <Glu-Gly-Arg-*p*NA makes it possible also to measure the effect when thrombin was added to induce polymerization (Petersen et al., 1988). The enhancement was maximal even at 0.2 μM polymerized fibrin (results not shown).

Effect of Lys_{416} Substitution on t-PA-Catalyzed Lys_{78}-Plasminogen Activation. The activity of authentic one-chain t-PA with Lys_{78}-plasminogen is only about 3% of that of two-chain t-PA (Petersen et al., 1988). Activity measurements with two-chain t-PA are relatively straightforward, whereas with one-chain t-PA they are complicated by the fact that the plasmin generated is capable of back-activation to generate two-chain t-PA during the assay. Figure 5 shows measurements of plasminogen activation catalyzed by authentic t-PA and [K416S,H417S]t-PA. After its conversion to the two-chain form, the activity of the analogue was 50% of that of authentic two-chain t-PA (closed symbols). Activity measurements with one-chain t-PA (open symbols) indicate a low initial activity in particular with one-chain [K416S,H417S]-t-PA; however, for the above-mentioned reason, only a qualitative estimation is possible. Two-chain generation was in fact indicated by a progressive increase in the slope of the curves.

Figure 6 shows Lineweaver–Burk plots of plasminogen activation with the two-chain forms of authentic t-PA and the two Lys_{416} substitution analogues. The k_{cat} but not K_m was changed significantly by the substitution.

Fibrin-Enhanced Lys_{78}-Plasminogen Activation. As has been shown by several groups (Tate et al., 1987; Petersen et al., 1988; Boose et al., 1989), t-PA analogues resistant to plasmin cleavage are capable of activating plasminogen at considerable rates. Apparently, fibrin binding induces an active conformation of one-chain t-PA. Results with [R275G]t-PA and [R275L,K277L]t-PA (not shown) confirm this and also suggest that this effect of fibrin does not depend on the presence of Lys_{277} in t-PA. Figure 7 shows progress curves obtained with the analogues [K416S]t-PA and [K416S,H417T]t-PA. Although the activity of these analogues was somewhat lower than that of authentic t-PA, it is clear that fibrin enhancement was not impaired by the substitution of Lys_{416}. Conversion into the two-chain counterparts of these analogues by plasmin incubation, prior to plasminogen activation measurements, did not change the kinetics significantly (results not shown).

Discussion

Trypsinogen activation induced by cleavage of a peptide bond between Lys_{15} and Ile_{16} results in salt-bridge formation between the α-amino group of Ile_{16} and the γ-carboxy group of Asp_{194} [see, e.g., Kraut (1977)]. As revealed by X-ray diffraction studies, this event is accompanied by structural changes that involve certain parts of the polypeptide backbone characterized by a disordered state in trypsinogen (Bode et al., 1976). Salt-bridge formation leads to strengthening of this flexible structure. It has been shown that a similar structural change leading to the "trypsin-like" conformation can be induced on the zymogen without cleavage of the Lys_{15}–Ile_{16}

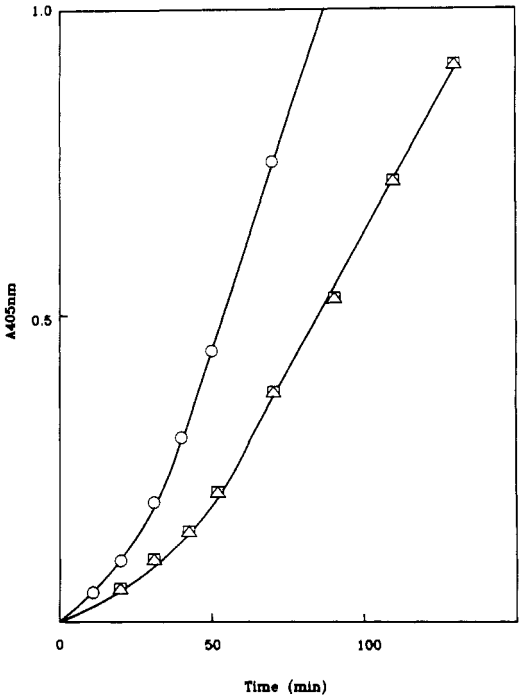

FIGURE 7: Effect of Lys_{416} substitution of plasminogen activation in the presence of fibrin. Progress curves for the generation of *p*NA. The activity of the substitution analogues [K416S]t-PA (△) and [K416S,H417T]t-PA (□) was compared to that of authentic t-PA (○). Additions: 0.06 nM t-PA, 0.2 μM Lys_{78}-plasminogen, 0.2 μM aprotinin, 0.6 mM Val-Phe-Lys-*p*NA, 0.15 μM fibrinogen, and 0.2 NIH unit/mL thrombin.

peptide bond. This may occur when trypsinogen forms a complex with aprotinin (Bode et al., 1978), or with synthetic peptides sequentially related to the Ile_{16} N-terminal of trypsin (Bode, 1979).

To explain this behavior, Bode (1979) suggested a model in which the "activation domain" of trypsinogen could exist either in a disordered, nonfunctioning, or in an ordered active state. It was further assumed that for trypsinogen the equilibrium between both conformations was strongly in favor of the disordered state ($K_{eq} = 10^8$), unless the ordered state was stabilized by high-affinity ligands. He also supposed that in other serine protease zymogens this conformation equilibrium could well be significantly shifted toward the active state.

As indicated by the present work and by several other studies, t-PA is in fact active also as the one-chain zymogen form. Thus, one-chain t-PA may provide an example of a zymogen in which the nonfunctioning and the active conformations are more equally balanced than in trypsinogen. A tentative hypothesis for the structural basis of this peculiarity may be suggested based on a comparison with serine proteases of known tertiary structure (Table I). Table I indicates that the amino acid sequences which constitute the spatial surroundings of Asp_{194} of chymotrypsinogen, trypsin, elastase, and kallikrein are highly conserved. The residues present within a radius of 8 Å from the α-carbon of Asp_{194} are indicated by the shaded areas. On the basis of an assumed homology of the tertiary structure of the t-PA protease domain to these proteases, it is reasonable to expect that a potential alternative salt-bridge formation in the t-PA zymogen would involve a positively charged residue confined to these sequences. Whereas no such residues are present in the sequences emphasized in chymotrypsinogen, trypsin, elastase, and kallikrein, two lysine residues of t-PA are likely to be close enough to Asp_{194} (Asp_{477} of the t-PA numbering) to potentially interact with this residue in one-chain t-PA.

The lysine residues highlighted by this argumentation (Lys_{277} and Lys_{416}) have previously been proposed as possible candidates for salt-bridge interaction, respectively, by Wallén et al. (1983) and by reference to a computer-graphic model (Heckel & Hasselbach, 1988). The results of the present study support the suggestion based on this model that such an interaction could be an important requirement for the amidolytic activity of one-chain t-PA. As expected, substitution of Lys_{416} with Ser leads to substantial quenching of the one-chain t-PA activity as indicated by a 5-fold reduction of k_{cat} and a 12-fold reduction in k_{cat}/K_m (Table II), whereas the activity of two-chain t-PA was much less affected. The maximal turnover (k_{cat}) was reduced 2-fold, but the apparent second-order constant (k_{cat}/K_m) for the reaction between enzyme and substrate was unchanged. Substitution of Lys_{416} with Ser also reduced the activity of t-PA with plasminogen as the substrate in the absence of fibrin (Figure 5). Apparently, the quenching was relatively more pronounced with one-chain t-PA than with two-chain t-PA, although continuous conversion to the two-chain form by the reaction product presents a technical difficulty in interpretation of these data.

Lys_{416} may contribute significantly to the stabilization of an active conformation of one-chain t-PA; however, its presence is probably not an absolute requirement for obtaining one-chain activity. This is indicated by several observations. The amidolytic activities of one-chain [K416S]t-PA and [K416S,H417T]t-PA, although low, are measurable, and it cannot be accounted for by the presence of two-chain trace impurities in the one-chain preparation. In this case, the amidolytic activity would have been characterized by a K_m value identical with that obtained for two-chain t-PA. The K_m values found for the one-chain form of these analogues are clearly much higher than the K_m values found when they are converted into their two-chain counterparts (Table II). The observation of fibrin-enhanced one-chain activity is also indicative of an active conformation which can be induced without involvement of the positive charge of Lys_{416}. Finally, the analogues are also capable of reacting with plasminogen activator inhibitor 1 (PAI-I) (results not shown).

These observations are readily accounted for by the conformation equilibrium model for zymogen activation proposed by Bode (1979). We believe that Lys_{416} provides an important contribution to the stabilization of an ordered functional state of one-chain t-PA. On the other hand, it is plausible that strong ligands such as fibrin and PAI-I may stabilize the active conformational state even more efficiently and thus compensate for the destabilization induced by site-specific alteration of Lys_{416}.

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