A Site-Directed Mutagenesis of Pro-urokinase Which Substantially Reduces Its Intrinsic Activity†

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ABSTRACT: Single-chain urokinase-type plasminogen activator or pro-urokinase is a zymogen with an intrinsic catalytic activity which is greater than that of most other zymogens. To study the structural basis for this activity, a three-dimensional homology model was calculated using the crystallographic structure of chymotrypsinogen, and the structure−function relationship was studied using site-directed mutagenesis and kinetic analysis. This model revealed a unique Lys^{300} in pro-urokinase which could form a weak interaction with Asp^{355}, adjacent to the active site Ser^{356}. It was postulated that this lysine, by its ϵ-amino group, may serve to pull Ser^{356} close to the active position, thereby inducing the higher intrinsic activity of pro-urokinase. This was consistent with the published finding that a homologous lysine (Lys^{416}) in single chain tissue plasminogen activator when mutated to serine induced some reduction in activity. To test this hypothesis, a site-directed mutant with a neutral residue ($Lys^{300} \rightarrow$ Ala) was produced and characterized. The Ala^{300}-pro-urokinase had a 40-fold lower amidolytic activity than that of pro-urokinase. It was also stable in plasma at much higher concentrations than pro-urokinase, reflecting much attenuated plasminogen activation. Plasmin activatability was comparable to that of pro-urokinase, but the resultant two-chain derivative (Ala^{300}-urokinase) had a lower enzymatic activity (≈33% that of urokinase) due to a reduction of k_{cat}. Interestingly, the K_M of two-chain Ala^{300}-urokinase against plasminogen was 5.8-fold lower than that of urokinase, being similar to that of pro-urokinase which has a K_M about 5-fold lower than urokinase. In conclusion, the hypothesis that Lys^{300} is a key structural determinant of the high intrinsic activity of pro-urokinase was confirmed by these studies. This residue also appears to be important for the full expression of the enzymatic activity of urokinase.

Single chain urokinase-type plasminogen activator or pro-urokinase (pro-UK)[1] and single chain tissue plasminogen activator (sc-t-PA) are physiological activators of plasminogen which have quite different catalytic properties. Whereas sc-t-PA is an enzyme (Rijken *et al.*, 1982), pro-UK is a zymogen which, in contrast to sc-t-PA, is nonreactive and stable in plasma at physiological concentrations (Pannell & Gurewich, 1986). However, its intrinsic activity is relatively high, corresponding to about 0.4% of UK activity against plasminogen (Pannell & Gurewich, 1987). This measurement is complicated by a positive feedback from the activation of pro-UK by plasmin, which has resulted in a wide range of values having been reported from a potency equivalent to UK (Collen *et al.*, 1986) to no measurable activity at all (Husain, 1991). Nevertheless, there is a consensus that the intrinsic activity of pro-UK is quantitatively distinct from other zymogens of the serine protease family (Petersen *et al.*, 1988; Lijnen *et al.*, 1990).

The intrinsic activity of pro-UK also has some qualitatively distinct properties such as its reversible inactivation by diisopropyl fluorophosphate (Liu & Gurewich, 1995). Secondly, pro-UK has a K_M against plasminogen lower than that of its two-chain enzymatic form, UK (Collen *et al.*, 1986; Liu & Gurewich, 1992, 1993; Liu *et al.*, 1992). This property is believed to be responsible for a hyperactive transitional state found during the conversion of pro-UK to UK in the presence of plasminogen, corresponding to the K_M of pro-UK and the k_{cat} of UK (Liu *et al.*, 1992). Finally, although pro-UK induces fibrin-specific clot lysis equivalent to that of t-PA, its mode of action is totally different (Pannell *et al.*, 1988; Liu & Gurewich, 1992). Whereas t-PA has a strong affinity to the D-domain of fibrin, which is responsible for its fibrin-dependent plasminogen activation, pro-UK has no fibrin affinity and its fibrin dependence is promoted by the E-domain of fibrin and has been related to a specific conformational change in plasminogen (Liu & Gurewich, 1991). In the presence of fragment E, the intrinsic activity of pro-UK against plasminogen is promoted 500-fold, giving it a catalytic efficiency equivalent to that of two-chain UK (Liu & Gurewich, 1992).

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[1] Abbreviations: pro-UK, pro-urokinase; UK, urokinase; DFP, diisopropyl fluorophosphate; sc-t-PA, single chain tissue plasminogen activator; tc-t-PA, two-chain tissue plasminogen activator; 3-D, three dimensional; OD, optical density.

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These unusual properties of the intrinsic catalytic domain of pro-UK prompted the present analysis of its structural determinants. A three-dimensional model of the protease domain of pro-UK was calculated by comparative protein modeling using the computer program MODELLER[2] (Sali & Blundell, 1993, 1994). The model relied on the crystallographically determined structure of chymotrypsinogen (Wang *et al.*, 1985). Certain site-directed mutants were made and characterized to validate the model. Another goal of these mutants was to improve the plasma inertness of pro-UK by reducing its intrinsic activity, so that at therapeutic concentrations it would be less apt to induce nonspecific plasminogen activation (PRIMI Trial Study Group, 1989).

MATERIALS AND METHODS

Materials

Recombinant wild-type pro-UK produced from *Escherichia coli* was obtained from Farmitalia Carlo Erba (Milan, Italy). Trace (<1%) two-chain UK contaminant was inactivated by treatment with DFP as previously described (Liu & Gurewich, 1995). The concentration of UK or pro-UK was standardized with the International Reference Preparation (National Institute for Biological Standards and Controls, London, U.K.). For this purpose, pro-UK was first converted to UK by incubation with plasmin as previously described (Pannell & Gurewich, 1987).

Glu-plasminogen was purified from DFP-treated bank plasma essentially by the method of Castellino and Powell (1981). Bank plasma was from the blood bank of Deaconess Hospital. Fibrinogen and synthetic chromogenic substrates for plasmin (S2251) and for UK (S2444) were obtained from Kabi (Franklin, OH). Lys-plasmin was obtained from American Diagnostica Inc. (Greenwich, CT). Fibrin fragment E was prepared as previously described (Liu & Gurewich, 1992). Thrombin was from Parke-Davis (Morris Plains, NJ). Enzymes and reagents for gene manipulation and expression of pro-UK were purchased from New England BioLabs (Beverly, MA) and Boehringer Mannheim (Indianapolis, IN). S-Sepharose, Sephadex G-25, benzamindine−Sepharose, aprotinin, and aprotinin−Sepharose were from Sigma (St. Louis, MO).

Methods

(1) 3-D Model of the Protease Domain of Pro-UK. A 3-D model of the protease domain (147−411) of pro-UK was built by MODELLER-1, a program that implements comparative modeling by satisfaction of spatial restraints (Sali & Blundell, 1993, 1994). The method is only briefly outlined here. The multiple alignment of pro-UK with serine proteases of known 3-D structures indicated that the crystallographic structure of bovine chymotrypsinogen (Brookhaven Protein Data Bank code 2CGA) (Wang *et al.*, 1985) was a suitable template structure for comparative modeling of the protease domain of pro-UK. Due to the relatively high similarity (33% identity) between the amino acid sequences of chymotrypsinogen (245 residues) and the protease domain of pro-UK (264 residues), the alignment was prepared by hand. The sequence alignment between chymotrypsinogen and pro-UK was used with MODELLER-1 to produce a model of the pro-UK protease domain containing all main chain and side chain heavy atoms without any further manual intervention. MODELLER-1 was first used to derive distance and dihedral angle restraints on the pro-UK sequence from its alignment with the template chymotrypsinogen structure. The spatial restraints and CHARMM-22 energy terms (Brooks *et al.*, 1983) enforcing proper stereochemistry were then combined into an objective function. The variable target function procedure was used to obtain 3-D models by optimizing the objective function. Ten slightly different 3-D models of the pro-UK protease domain were calculated by varying the initial structure. The root-mean-square difference for superposition of the main chain atoms of these models was generally less than 0.2 Å. The structure with the lowest value of the objective function was selected as the representative model (Figure 1A). The model of the pro-UK protease domain passed all the stereochemistry checks implemented in the program PROCHECK-3 (Laskowski *et al.*, 1993).

(2) Gene Construction, Expression, and Purification of Ala^{300}- and Glu^{303}-pro-UK. The gene for native pro-UK has been well characterized (Verde *et al.*, 1984), and its cDNA was available from Dr. Paolo Sarmientos (Farmitalia, Milano, Italy). The site-directed mutants (Lys^{300} → Ala and Ser^{303} → Glu) of pro-UK were constructed and expressed in *E. coli* as follows: The cDNA of the pro-UK mutant (Lys^{300} → Ala and Ser^{303} → Glu) was obtained by site-directed mutagenesis after subcloning the *Hin*dIII−*Bam*HI restriction fragment from pFC16 plasmid containing the full-length cDNA of pro-UK (Orini *et al.*, 1991) into an M13 vector (mp18). A specific synthetic oligonucleotide (5′-TGGCTTTG-GAGCGGAGAATTCTAC for Ala^{300} and 5′-GAAAA-GAGAATGAAACCGACTAT for Glu^{303}) coding for the designed site mutant was first hybridized to the recombinant M13 vector and then extended with T4 DNA polymerase. Following ligation and transformation of bacterial cells, positive clones were selected for DNA sequencing. The expression plasmid for the mutant was constructed by reinserting the mutated *Hin*dIII−*Bam*HI fragment in pFC16 and introduced into an *E. coli* type B strain. Ala^{300}- or Glu^{303}-pro-UK was purified from sonicated cell lysates by chromatography through an S-Sepharose, pro-UK antibody affinity column and Sephadex G-25 after refolding by the method previously described (Winkler & Blaber, 1986). Trace amounts of UK were removed by passage over benzamidine−Sepharose followed by treatment with DFP as previously described (Liu & Gurewich, 1995). Purified Ala^{300}- or Glu^{303}-pro-UK was observed as single bands on reduced SDS−PAGE. Protein concentration was determined from absorbance at 280 nm using the extinction coefficient ($E^{1\%}_{280nm} = 1.36$) for pro-UK.

(3) Assay of Plasmin Sensitivity: A Kinetic Study of Pro-UK or Ala^{300}-pro-UK Activation by Lys-plasmin. Pro-UK or Ala^{300}-pro-UK at a range of concentrations (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 2.5, 3.5, and 5.0 μM) was incubated with Lys-plasmin (0.1 nM) in the presence of 1.2 mM S2444 (UK synthetic substrate) in 0.05 M Tris-HCl, 0.10 M NaCl, and 0.01% Tween 80, pH 7.4 at room temperature over time. Pro-UK/mutant without plasmin at the same range of concentrations was incubated with S2444 as control. The 0.1 nM plasmin was shown to have no direct

[2] MODELLER is available by anonymous FTP from tammy.harvard.edu:pub/modeler and also as part of QUANTA (MSI, Burlington, MA. E-mail: jcollins@msi.com).

effect on S2444 hydrolysis under the experimental conditions. The rate of pro-UK activation was calculated from the OD increase over time squared at 410 nm against a reference wavelength of 490 nm (410/490 nm) on a microtiter plate reader (MR 5000; Dynatech Laboratories, Inc., Alexandria, VA) as previously described (Liu & Gurewich, 1991; Liu & Gurewich, 1993). The kinetic constants were derived by Lineweaver−Burk analysis.

(4) Intrinsic Activity Assay. (A) Hydrolysis of S2444. Pro-UK (1.0 μM), Glu^{303}-pro-UK (1.0 μM), or Ala^{300}-pro-UK (10.0 μM) was incubated with 0.6 mM S2444 in 0.05 M sodium phosphate, 0.10 M NaCl, 0.2% BSA, and 0.01% Tween 80 (pH 7.4) at room temperature. The reaction rate was measured by the linear OD increase over time at 410 nm against a reference wavelength of 490 nm (410/490 nm) on the microtiter plate reader. UK International Standards (0.01−5.0 nM) were used for the standard curve of S2444 activity of UK.

(B) Plasma Inertness. Ala^{300}-pro-UK (0−50 μg/mL), Glu^{303}-pro-UK (0−10 μg/mL), or pro-UK (0−10 μg/mL) was incubated in 1.0 mL of pooled bank plasma at 37 °C for 6 h, after which time 0.2 mL of aprotinin (10 000 KIU/mL) was added and the plasma fibrinogen remaining was measured by the thrombin-clottable protein method (Swaim & Feders, 1967).

(5) Activities of Two-Chain UK or Ala^{300}-UK. (A) Hydrolysis of S2444. UK or Ala^{300}-UK was prepared by plasmin treatment of single chain precursors as previously described (Pannell & Gurewich, 1987). Plasmin was removed with aprotinin−Sepharose. UK or Ala^{300}-UK (4.0 nM) was incubated with a range of concentrations (0.03, 0.06, 0.12, 0.18, 0.24, 0.3, 0.6, 1.2, 1.8, and 2.4 mM) of S2444 in 0.05 M sodium phosphate, 0.10 M NaCl, 0.2% BSA, and 0.01% Tween 80 (pH 7.4) at room temperature. The reaction rate was measured as described above.

(B) Glu-plasminogen Activation. Time−absorbance curves of Glu-plasminogen activation were obtained by measuring the OD increase of the reaction mixture with time at the selected wavelength, 410 nm, and reference wavelength, 490 nm (410/490 nm), on a microtiter plate reader (Dynatech MR 5000). The reaction mixture contained S2251 (plasmin synthetic substrate) (1.5 mM), Glu-plasminogen (1.0, 1.5, 2.5, 3.5, 4.5, 5.5, 7.5, and 10.0 μM), and UK or Ala^{300}-UK (0.2 nM). The reactants were made up in 0.05 M sodium phosphate, 0.10 M NaCl, 0.2% BSA, and 0.01% Tween-80, pH 7.4, and incubated at room temperature. The reaction rates were calculated from the OD increasing over time squared as previously described (Liu & Gurewich, 1993).

The kinetic constants were calculated by Lineweaver−Burk plots.

(6) Promotion of Plasminogen Activation by Fibrin Fragment E-2. Since fibrin fragment E was shown to selectively (Liu & Gurewich, 1991) and potently (Liu & Gurewich, 1992) promote Glu-plasminogen activation by pro-UK, the promotion by fragment E-2 of this reaction catalyzed by Ala^{300}-pro-UK was analyzed and compared with that obtained with pro-UK. This was determined as previously described (Liu & Gurewich, 1992) by measuring the OD increase with time in the reaction mixture at 410 nm against a reference wavelength of 490 nm (410/490 nm) on the microtiter plate reader. The reaction mixture contained 1.5 mM S2251, Glu-plasminogen (2.0 μM), and 2.0 nM pro-UK or Ala^{300}-pro-UK with or without 5.0 μM fragment E_2, in 0.05 M sodium phosphate, 0.10 M NaCl, 0.2% BSA, and 0.01% Tween 80, pH 7.4 at room temperature.

(7) Fibrin Clot Lysis. ^{125}I-Labeled clots were prepared from 0.25 mL of plasma as previously described (Gurewich *et al.*, 1984). Clot lysis experiments were performed in 3 mL of plasma with a range of concentrations of pro-UK or Ala^{300}-pro-UK (0.5, 0.75, 1.25, 1.5, 2, 2.5, 3.0, 4.0, 5.0, 7.5, and 10 μg/mL) or sc-t-PA (5, 10, 30, 50, 75, 100, and 150 ng/mL) and certain combinations of sc-t-PA and pro-UK/mutant. Lysis was quantitated from the release of radioactivity and expressed as a percent of the complete lysis value against time.

RESULTS AND DISCUSSION

Pro-urokinase is inert in plasma at physiological concentrations and does not form SDS-stable complexes with inhibitors (Pannell & Gurewich, 1986). However, when incubated with plasminogen in the absence of inhibitors, it readily generates plasmin after a concentration-dependent lag phase (Pannell & Gurewich, 1987). This activity is initiated by the relatively high intrinsic activity (0.2−0.4%) of pro-UK, which is substantially higher than that of other protease zymogens (Robinson *et al.*, 1973; Gertler *et al.*, 1974). After plasminogen activation has been initiated by pro-UK, plasmin generation is amplified by the conversion of pro-UK to UK by plasmin. In plasma, these reactions are confined to the fibrin surface by the plasma protease inhibitors, since plasminogen activation by pro-UK is dramatically promoted by fibrin and is not significantly affected by the inhibitors. At higher concentrations of pro-UK in plasma, fibrin-independent plasminogen activation is triggered by its relatively high intrinsic activity. This nonspecific plasminogen activation is amplified by the systemic conversion of pro-UK to UK, resulting in further loss of fibrin specificity (Koster *et al.*, 1994). Therefore, a mutant pro-UK with a lower intrinsic activity should have the potential of being a more fibrin-specific thrombolytic agent.

(1) Model Analysis and Hypothesis. Since the X-ray crystallography structure of pro-UK has not yet been reported, the serine protease domain of pro-UK was modeled by relying on its 33% sequence identity to chymotrypsinogen (Table 1), whose structure has been determined by X-ray crystallography (Wang *et al.*, 1985). In trypsinogen, the electrostatic interaction of the newly formed amino terminus with the Asp next to the active site Ser is part of the zymogen activation that results in the fully active enzyme (Walter *et al.*, 1982). The inspection of the 3-D model of pro-UK indicated that the intrinsic activity may result from a functional replacement of the neo-amino terminus in trypsinogen by Lys^{300} in pro-UK. In other words, it was speculated that the Lys^{300}−Asp^{355} interaction induces the activated active site conformation, just as the native neo-amino terminus does in trypsin.

It was further postulated that the Lys^{300}−Asp^{355} interaction is made possible by flexibility of the Lys^{300} loop (residues 297−313). The proposed flexible nature of the Lys^{300} loop in pro-UK is based on homologous regions in the crystallographic structures of trypsinogen (Walter *et al.*, 1982) and chymotrypsinogen (Wang *et al.*, 1985) that have very high isotropic temperature factors, indicating that they are probably flexible in solution. Most of the time, the flexible loop of pro-UK is in an "inactive conformation" in which the

Table 1: Sequence Alignment of Serine Proteases[a]

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                          16  20        30              40        50
2cga      CGVPAI-QPVLSGLSRIVNGEEAVPGSWPWQVSLQDKT-----GFHFCGGSLINENWVVT(54)
1ton      ----------------IVGGYKCEKNSQPWQVAVINE--------YLCGGVLIDPSWVIT
2pka      ----------------IIGGRECEKNSHPWQVAIYHY------SSFQCGGVLVNPKWVLT
2ptn      ----------------IVGGYTCGANTVPYQVSLNS-------GYHFCGGSLINSQWVVS
3est      ----------------VVGGTEAQRNSWPSQISLQYRS--GSSWAHTCGGTLIRQNWVMT
1hne      ----------------IVGGRRARPHAWPFMVSLQLR------GGHFCGATLIAPNFVMS
1sgt      ----------------VVGGTRAAQGEFPFMVRLS----------MGCGGALYAQDIVLT
ukhu      CGQK---TLRPR--FKIIGGEFTTIENQPWFAAIYRRHR-GGSVTYVCGGSLMSPCWVIS(201)
ukhut     CGLRQY-S-QPQ--FRIKGGLFADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWILS(319)
               60              70        80        90              100
2cga      AAHCGVT-----TSDVVVAGEFDQGSSSEKIQKLKIAKVFKNSKYNSL----TI--NNDI(103)
1ton      AAHCYSN------NYQVLLGRNNLFKDEPFAQRRLVRQSFRHPDYIPLP-VHDH--SNDL
2pka      AAHCKND------NYEVWLGRHNLFENENTAQFFGVTADFPHPGFNLSADGKDY--SHDL
2ptn      AAHCYKS------GIQVRLGEDNINVVEGNEQFISASKSIVHPSYNSN----TL--NNDI
3est      AAHCVDR----ELTFRVVVGEHNLNQNNGTEQYVGVQKIVVHPYWNTD----DVAAGYDI
1hne      AAHCVAN--VNVRAVRVVLGAHNLSRREPTRQVFAVQRIFED-GYDPV----NL--LNDI
1sgt      AAHCVSG-SGNNTSITATGGVVDLQS--GAAVKVRSTKVLQAPGYNGT--------GKDW
ukhu      ATHCFID-YPKKEDYIVYLGRSRLNSNTQGEMKFEVENLILHKDYSAD----TLAHHNDI(256)
ukhut     AAHCER---FPPHHLTVILGRTYRVVPGEEEQKFEVEKYIVHKEFDDD----TY--DNDI(370)
                110             120       130       140          150
2cga      TLLKLSTA------ASFSQTVSAVCLPSASDDFAAGTTCVTTGWGLTRY--TNANTPDRL(155)
1ton      MLLHLSEP------ADITGGVKVIDLP--TKEPKVGSTCLASGWGSTNP--SEMVVSHDL
2pka      MLLRLQSP------AKITDAVKVLELP--TQEPELGSTCEASGWGSIEPGPDDFEFPDEI
2ptn      MLIKLKSA------ASLNSRVASISLP--TSCASAGTQCLISGWGNTKS--SGTSYPDVL
3est      ALLRLAQS------VTLNSYVQLGVLPRAGTILANNSPCYITGWGLTRT---NGQLAQTL
1hne      VILQLNGS------ATINANVQVAQLPAQGRRLGNGVQCLAMGWGLLGR---NRGIASVL
1sgt      ALIKLAQP------IN----QPTLKIA--TTTAYNQGTFTVAGWGANRE---GGSQQRYL
ukhu      ALLKIRS-KEGRCA-QPSRTIQTICLPSMYNDPQFGTSCEITGFGKENS--TDYLYPEQL(312)
ukhut     ALLQLKS-DSSRCA-QESSVVRTVCLPPADLQLPDWTECELSGYGKHEA--LSPFYSERL(428)
                                                      ↑
              160       170                 180               190
2cga      QQASLPLLSNTNCKK--YWGTKIK---------DAMICAGA-------S-GVSSCMGDSGGP(198)
1ton      QCVNIHLLSNEKCIE--TYKDNVT---------DVMLCAGEM------EGGKDTCAGDSGGP
2pka      QCVQLTLLQNTFCAD--AHPDKVT---------ESMLCAGYL------PGGKDTCMGDSGGP
2ptn      KCLKAPILSDSSCKS--AYPGQIT---------SNMFCAGYL------EGGKDSCQGDSGGP
3est      QQAYLPTVDYAICSSSSYWGSTVK---------NSMVCAGG-------DGVRSGCQGDSGGP
1hne      QELNVTVV-TSLC---------R----------RSNVCTLVR------GRQAGVCFGDSGSP
1sgt      LKANVPFVSDAACRS--AYGNELV---------ANEEICAGYP-----DTGGVDTCQGDSGGP
ukhu      KMTVVKLISHRECQQPHYYGSEVT---------TKMLCAADP------QWKTDSCQGDSGGP(359)
ukhut     KEAHVRLYPSSRCTSQHLLNRTVT---------DNMLCAGDTRSGGPQANLHDACQGDSGGP(481)
           200       210       220       230       240
2cga      LVCKKN-GAWTLVGIVSWGSST-C-STSTPGVYARVTALVNWVQQTLAAN-----
1ton      LICD-----GVLQGITSGGA-TPCAKPKTPAIYAKLIKFTSWIKKVMKENP----
2pka      LICN-----GMWQGITSWGH-TPCGSANKPSIYTKLIFYLDWIDDTITENP----
2ptn      VVCS-----GKLQGIVSWGS--GCAQKNKPGVYTKVCNYVSWIKQTIASN-----
3est      LHCLVN-GQYAVHGVTSFVSRLGCNVTRKPTVFTRVSAYISWINNVIASN-----
1hne      LVCN-----GLIHGIASFVR-GGCASGLYPDAFAPVAQFVNWIDSIIQ-------
1sgt      MFRKDNADEWIQVGIVSWGY--GCARPGYPGVYTEVSTFASAIASAARTL-----
ukhu      LVCSLQ-GRMTLTGIVSWGR--GCALKDKPGVYTRVSHFLPWIRSHTKEENGLAL
ukhut     LVCLND-GRMTLVGIISWGL--GCGQKDVPGVYTKVTNYLDWIRDNMRP------
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[a] 1ton, tonin; 2pka, kallikrein A; 2ptn, trypsin; 2cga, chymotrypsinogen; 3est, pancreatic elastase; 1hne, neutrophil elastase; 1sgt, trypsin; ukhu, pro-urokinase B-chain; ukhut, tissue plasminogen activator B-chain. The Lys[300] (ukhu) and Lys[416] (ukhut)are indicated by the arrow.

positively charged ϵ-amino group of Lys[300] does not interact with the negatively charged carboxyl group of Asp[355] (as modeled in Figure 1A). It is postulated that the considerable movement of the flexible loop approximates these residues transiently, inducing an "active conformation" in pro-UK without conversion to two-chain UK (as illustrated in Figure 1B). The concept that pro-UK can assume a fully active conformation (Figure 1B) is supported by the observation that, in the presence of fragment E, the catalytic efficiency of pro-UK against Glu-plasminogen is equivalent to that of two-chain UK (Liu & Gurewich, 1992). It is also consistent with the hypothesis that a homologous lysine residue (Lys[416]) was found in sc-t-PA (Table 1) which has an even higher single chain activity, being 15−22% of the two-chain-t-PA (tc-t-PA) against S2888 (Tate *et al.*, 1986; Petersen *et al.*, 1990; Madison *et al.*, 1993). This lysine was thought to be responsible for the high intrinsic activity of sc-t-PA based on a homology model derived from the known structure of elastase (Heckel & Hasselbach, 1988). A subsequent experimental study showed that a Lys[416] mutation was associated with a reduction in activity, but it is now believed that the absence of the zymogen triad is the major determinant for the high intrinsic activity of sc-t-PA (Madison *et al.*, 1993).

To test the hypothesis that the positive charged side chain of Lys[300] is involved in the high intrinsic activity of pro-UK, a site-directed mutant of Lys[300] → Ala was made in *E. coli*. As a control, another close site mutant, Ser[303] → Glu, unrelated to any interaction with the active site of the enzyme, was also produced in order to determine whether

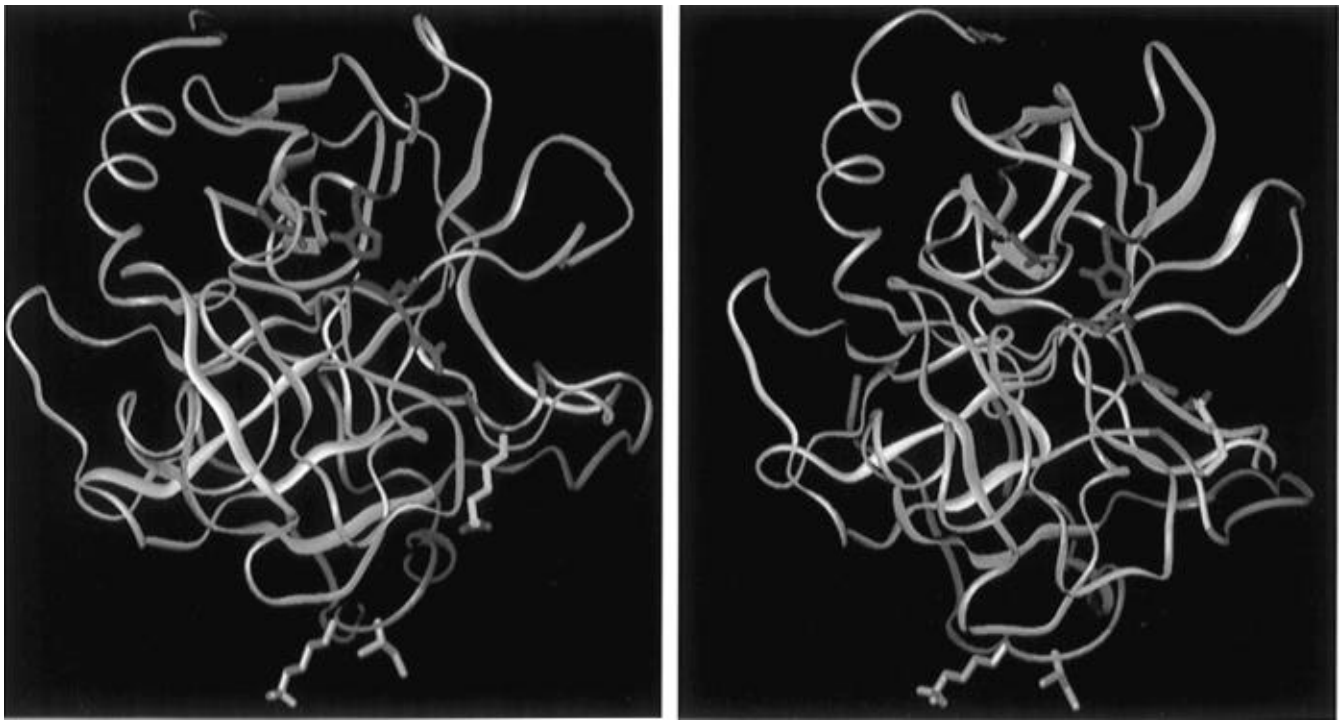

FIGURE 1: Three-dimensional model of the protease domain of pro-UK showing the inactive conformation (A, left) and active conformation (B, right). The flexible loop is in blue and Lys^{300} is in yellow. The ϵ-amino group of Lys^{300} (red) is shown to interact with Asp^{355} (green) only in the active conformation (B). The active site residues are shown in red, and the activation residues are in pink. The model in (B) was calculated from the model in (A) using Charmm with constraints to illustrate the possible interaction between Lys^{300} and Asp^{355}. Quanta (Molecular Simulations, Burlington, MA) was used to display this model.

mutations in this region affected the activity of pro-UK.

(2) Intrinsic Activities of Ala^{300} and Glu^{303}-pro-UK. The intrinsic activity of Ala^{300}-pro-UK against synthetic substrate was found to be much reduced and was determined to be equivalent to 0.51 ± 0.38 nM UK activity for 10.0 μM Ala^{300}-pro-UK, a ratio of ≈0.005%. This is to be compared with 1.9 ± 0.24 nM UK activity for 1.0 μM pro-UK, or ≈0.2%. Therefore, the intrinsic activity of pro-UK was reduced about 40-fold by a single site mutation at Lys^{300}. The intrinsic activity of Glu^{303}-pro-UK was 0.17%, similar to that of pro-UK, showing that mutations in this region do not nonspecifically interfere the pro-UK activity.

In sc-t-PA, a 12-fold reduction of activity was reported by a mutant of Lys^{416} → Ser (Petersen *et al.*, 1990). Therefore, the corresponding residue in pro-UK, Lys^{300}, plays a more critical role in the intrinsic activity of the single chain form. In sc-t-PA, the loss of the zymogenic triad was apparently more critical to the high intrinsic activity of sc-t-PA, since a mutant with the restored zymogenic triad (Ala^{292} → Ser and Phe^{305} → His) reduced its intrinsic activity by 42-fold to 0.38% of tc-t-PA (Madison *et al.*, 1993). This activity is still 76-fold higher than that (0.005%) of the Lys^{300}-pro-UK mutant. The zymogenic triad is generally not essential for the zymogenicity of serine proteases, since a number of serine proteases are zymogenic without this triad (Walter *et al.*, 1982). The importance of the zymogenic triad in pro-UK is unknown, but the present study shows that Lys^{300} is a major determinant of the intrinsic activity.

The intrinsic activity of the Ala^{300}-pro-UK against plasminogen could not be accurately measured directly due to its sensitivity to activation by plasmin (see below). Therefore, plasma inertness was used as a surrogate end point. It was also considered to be a clinically important property.

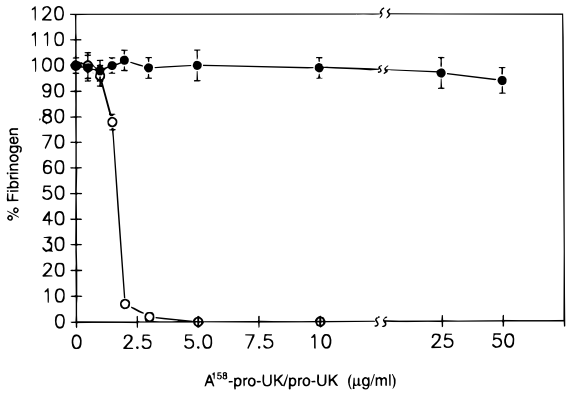

FIGURE 2: Inertness of Ala^{300}-pro-UK (●) or pro-UK (○) in plasma after incubation (37 °C) for 6 h.

(3) Plasma Inertness of Ala^{300}-pro-UK Compared with Pro-UK. Pro-UK is inert and stable in plasma only at concentrations at which nonspecific plasminogen activation is not triggered, leading to plasmin generation, consequent activation of pro-UK, and degradation of certain clotting factors like fibrinogen. Therefore, fibrinogen degradation was used as an indirect measure of the inertness of pro-UK in plasma.

After incubation (37 °C) in plasma for 6 h, pro-UK and Glu^{303}-pro-UK (data not shown) induced degradation of 100% of the plasma fibrinogen at concentrations ≥1 μg/mL. By contrast, Ala^{300}-pro-UK induced no fibrinogen degradation even at a concentration of 50 μg/mL, the highest concentration used in the experiment. Therefore, the mutant Ala^{300}-pro-UK was more than 50-fold more inert in plasma than pro-UK, consistent with its 40-fold lower intrinsic activity against synthetic substrate (Figure 2). These findings were of interest since a pro-UK which is less prone to induce systemic plasminogen activation should be a more fibrin-

Table 2: Kinetic Analysis of Ala^{300}-pro-UK or Pro-UK (0.1−5.0 μM) Activation by Lys-plasmin (0.1 nM)

	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M	F (%)
Ala^{300}-pro-UK	2.70 ± 0.42	52.69 ± 7.54	19.5	156
pro-UK	2.44 ± 0.57	30.39 ± 6.16	12.5	100

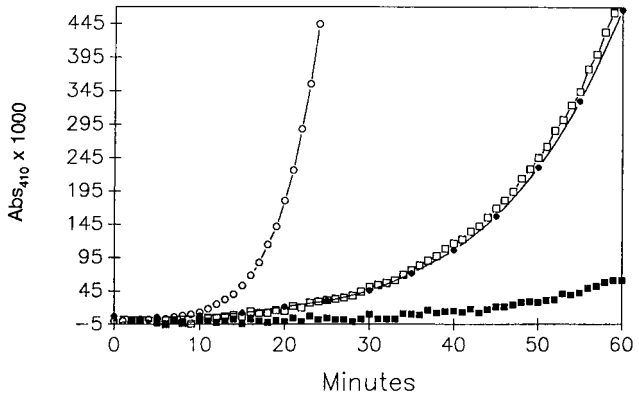

FIGURE 3: Pro-UK (○/●) or Ala^{300}-pro-UK (□/■) induced plasminogen activation in the presence (solid symbols) and absence (open symbols) of fragment E (5 μM).

Table 3: Kinetic Analysis of S2444 (0.03−2.4 mM) Hydrolysis by Ala^{300}-UK or UK (4.0 nM)

	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M	F (%)
Ala^{300}-tc-UK	77 ± 21	0.434 ± 0.054	0.00560	30
Tc-UK	77 ± 12	1.451 ± 0.216	0.01884	100

specific thrombolytic, assuming that its fibrinolytic properties were retained.

(4) Clot Lysis in a Plasma Milieu. The Ala^{300}-pro-UK was, however, found to have a much reduced fibrinolytic activity, even against sc-t-PA pretreated clots which have previously been shown to be more susceptible to lysis by pro-UK (Pannell *et al.*, 1988). The concentration of Ala^{300}-pro-UK needed to achieve a clot lysis rate equivalent to pro-UK was more than 10-fold greater. In contrast, Glu^{303}-pro-UK induced clot lysis comparable to that of pro-UK (data not shown).

These findings suggested an impairment of one or more of the following properties of pro-UK which have been shown to be important to its fibrinolytic mechanism of action: (1) plasmin activatability of the single chain form, (2) the catalytic activity of the two-chain form, (3) promotion of the intrinsic activity of the single chain form by fibrin fragment E.

(5) Plasmin Activatability of the Ala^{300}-pro-UK. The Ala^{300}-pro-UK was found to be slightly more sensitive (1.5-fold) to activation by plasmin than pro-UK. This change was found to be related entirely to an increase in the k_{cat} of the reaction (Table 2), suggesting that the flexible loop and Lys^{300} slightly influence proteolysis of the activation site at Lys^{158}−Ile^{159} by plasmin. This is consistent with the relatively close spatial relation between Lys^{300} and Lys^{158}−Ile^{159} (shown in pink) which was observed in the 3-D model of pro-UK (Figure 1).

(6) Activities of Two-Chain Ala^{300}-UK against Synthetic Substrate (Table 3) and against Glu-plasminogen (Table 4). When the activities of Ala^{300}-UK and UK were compared, the mutant was found to have a diminished catalytic efficiency, which was 30−33% that of UK against both

Table 4: Kinetic Analysis of Glu-plasminogen (1.0−10.0 μM) Activation by Ala^{300}-UK or UK (0.2 nM)

	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M	F (%)
Ala^{300}-tc-UK	3.30 ± 0.40	0.495 ± 0.072	0.15	33
Tc-UK	19.2 ± 4.1	8.65 ± 1.21	0.45	100

synthetic substrate (S2444) and Glu-plasminogen. In the case of the synthetic substrate, the difference in activity was related exclusively to the k_{cat}, which was reduced by about 3-fold. However, against plasminogen, the K_M of the mutant was actually 6-fold lower than that of UK, which was more than compensated for by a 17.5-fold reduction in the k_{cat}. The lower K_M suggests that the substrate binding pocket of the Ala^{300}-UK was better formed than that of UK. In this sense, the substrate binding pocket of Ala^{300} -UK resembles that of pro-UK rather than UK, since pro-UK has previously been shown also to have a K_M about 5-fold lower than that of UK (Liu & Gurewich, 1992).

These findings indicate that Lys^{300} is also involved in the formation of the active site of UK. An analogous observation was made with tc-t-PA where it was proposed that Lys^{416} interacted with Asp^{477}, the residue next to active site Ser^{478} (Nienaber *et al.*, 1992). The Lys^{416} → Ser tc-t-PA mutant was reported to have a 50% loss of activity against plasminogen and an about 20% loss against the synthetic substrate (Petersen *et al.*, 1990).

This interaction between Lys$^{300/416}$ of the flexible loop and the active site of UK/t-PA is unusual among serine proteases, since most of them have no homologous Lys and only require a salt bridge between the neo-amino terminus and the Asp next to the active site Ser for their full activities.

The lower catalytic efficiency of Ala^{300}-UK undoubtedly contributed to the impairment of clot lysis by Ala^{300}-pro-UK, since clot lysis by pro-UK is dependent, in large part, on the catalytic effect of UK (Gurewich *et al.*, 1988). However, since a more than 10-fold impairment in clot lysis was found, the 3-fold reduction in activity is probably not the only explanation.

(7) Promotion of Ala^{300}-pro-UK-Induced Plasminogen Activation by Fragment E. Since the fibrin promotion of pro-UK-induced plasminogen activation is dependent specifically (Liu & Gurewich, 1991) and potently (Liu & Gurewich, 1992) on fibrin fragment E, this reaction was tested with Ala^{300}-pro-UK. The promotion by fragment E_2 of plasminogen activation by Ala^{300}-pro-UK was significantly attenuated compared to that by pro-UK (Figure 3). The attenuation of this functionally important property, together with the lower catalytic activity of Ala^{300}-UK, probably accounts for the impairment of clot lysis by Ala^{300}-pro-UK.

The characterization of Ala^{300}-pro-UK confirms the prediction of the computer model regarding the contribution of the ϵ-amino group of the Lys^{300} residue to the high intrinsic activity of pro-UK. The properties of the Ala^{300}-pro-UK also confirm that the low plasminogen activator activity reported by most investigators for pro-UK (Pannell & Gurewich, 1987; Petersen *et al.*, 1988; Lijnen *et al.*, 1990; Liu & Gurewich, 1995) reflects its intrinsic activity and not trace UK contaminants, as recently suggested (Husain, 1991). The intrinsic activity of pro-UK against plasminogen is capable of being promoted 500-fold by fibrin fragment E by an unknown mechanism (Liu & Gurewich, 1992). The present

study suggests that this phenomenon may be structurally related to stabilization of the salt bridge between Lys^{300} and Asp^{355}.

The present study provides evidence for the importance of Lys^{300} to both the intrinsic activity of pro-UK and the enzymatic activity of UK, as shown by the 40-fold reduction in intrinsic activity and the attenuation (66%) of two-chain enzymatic activity when the positive charged side chain was eliminated. Interestingly, the Ala^{300}-UK had a 6-fold lower K_M than wild-type UK, being similar to that of pro-UK (Liu & Gurewich, 1992, 1993), suggesting structural similarities in the binding pockets of Ala^{300} UK and pro-UK. This finding is helpful in the design of mutant forms of u-PA in which both pro-UK's, K_M and k_{cat} are preserved.

The attenuation of the fragment E promotion of plasminogen activation by Ala^{300}-pro-UK suggests an interaction between the substrate, plasminogen, and the flexible domain of the enzyme. Such an interaction is also supported by the finding that plasmin activation of Ala^{300}-pro-UK was enhanced.

The role for the flexible loop as a part of the activation domain has been generally ignored in previous studies of serine proteases. Since this region is adjacent to the active site, it is possible that it has a direct functional importance which the present findings have shown.

In conclusion, elimination of the ϵ-amino group at position 300 of pro-UK reduced its intrinsic activity by about 40-fold, bringing it close to that of a typical protease zymogen. This finding is consistent with the hypothesis based on modeling that the relatively high intrinsic activity of pro-UK is related to partial stabilization of the flexible loop by a weak salt bridge between Lys^{300} and Asp^{355}, adjacent to the active site serine. The compromised fibrinolytic properties of the Lys^{300} mutant might be explained by the loss of two-chain activity and the attenuation of its fibrin fragment E promotion of plasminogen activator activity. As a result, the Lys^{300} mutation, while giving pro-UK the predicted and desirable improvement in plasma inertness, lost other properties important to the efficacy of a second generation pro-UK thrombolytic agent.

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REFERENCES

Brooks, B. R., Bruccoleri, R. E., Olafson, B. D., States, D. J., Swaminathan, S., & Karplus, M. (1983) *J. Comput. Chem. 4*, 187−217.

Castellino, F. J., & Powell, J. R. (1981) *Methods Enzymol. 80*, 365−378.

Collen, D., Zamarron, C., Lijnen, H. R., & Hoylaerts, M. (1986) *J. Biol. Chem. 261*, 1259−1266.

Gertler, A., Walsh, K. A., & Neurath, H. (1974) *Biochemistry 13*, 1302.

Gurewich, V., Pannell, R., Louie, S., Kelley, P., Suddith, R. L., & Greenlee, R. (1984) *J. Clin. Invest. 73*, 1731−1739.

Gurewich, V., Pannell, R., Broeze, R. J., & Mao, J. I. (1988) *J. Clin. Invest. 82*, 1956−1962.

Heckel, A., & Hasselbach, K. M. (1988) *J. Comput.-Aided Mol. Des. 2*, 7−14.

Husain, S. S. (1991) *Biochemistry 30*, 5797−5805.

Koster, R. W., Cohen, A. F., Hopkins, G. R., Beier, J., Gunzler, W. A., & van der Wouw, P. A. (1994) *Thromb. Haemostasis 72*, 740−744.

Laskowski, R. L., McArthur, M. W., Moss, D. S., & Thornton, J. M. (1993) *J. Appl. Crystallogr. 26*, 283−291.

Lijnen, H. R., Van Hoef, B., Nelles, L., & Collen, D. (1990) *J. Biol. Chem. 265*, 5232−5236.

Liu, J., & Gurewich, V. (1991) *J. Clin. Invest. 88*, 2012−2017.

Liu, J., & Gurewich, V. (1992) *Biochemistry 31*, 6311−6317.

Liu, J., & Gurewich, V. (1993) *Blood 81*, 980−987.

Liu, J., & Gurewich, V. (1995) *J. Biol. Chem. 270*, 8408−8410.

Liu, J., Pannell, R., & Gurewich, V. (1992) *J. Biol. Chem. 267*, 15289−15292.

Madison, E. L., Kobe, A., Gething, M.-J., Sambrook, J. F., & Goldsmith, E. J. (1993) *Science 262*, 419−421.

Nienaber, V. L., Young, S. L., Birktoft, J. J., Higgins, D. L., & Berliner, L. J. (1992) *Biochemistry 31*, 3852−3861.

Orini, G., Brandazza, A., Sarmientos, P., Molinari, A., Lansen, J., & Cauet, G. (1991) *Eur. J. Biochem. 195*, 691−697.

Pannell, R., & Gurewich, V. (1986) *Blood 67*, 1215−1223.

Pannell, R., & Gurewich, V. (1987) *Blood 69*, 22−26.

Pannell, R., Black, J., & Gurewich, V. (1988) *J. Clin. Invest. 81*, 853−859.

Petersen, L. D., Lund, L. R., Dano, K., Nielsen, L. S., & Skriver, L. (1988) *J. Biol. Chem. 263*, 11189−11195.

Petersen, L. C., Boel, E., Johannessen, M., & Foster, D. (1990) *Biochemistry 29*, 3451−3457.

PRIMI Trial Study Group (1989) *Lancet 1*, 863−868.

Rijken, D. C., Hoylaerts, M., & Collen, D. (1982) *J. Biol. Chem. 257*, 2920−2925.

Robinson, N. C., Neurath, H., & Walsh, K. A. (1973) *Biochemistry 12*, 420.

Sali, A., & Blundell, T. L. (1993) *J. Mol. Biol. 234*, 779−815.

Sali, A., & Blundell T. L. (1994) *Protein Sci. 3*, 1582−1596.

Swaim, W. R., & Feders, M. B. (1967) *Clin. Chem. 13*, 1026−1031.

Tate, K. M., Higgins, D. L., Holmes, W. E., Winkler, M. E., Heyneker, H. L., & Vehar, G. A. (1986) *Biochemistry 26*, 338−343.

Verde, P., Stoppelli, M. P., Galeffi, P., Di Nocera, P., & Blasi, F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4727−4731.

Walter, J., Steigemann, W., Singh, T. P., Bartunik, H., Bode, W., & Huber, R. (1982) *Acta Crystallogr. B38*, 1462−1472.

Wang, D., Bode, W., & Huber, R. (1985) *J. Mol. Biol. 185*, 595−624.

Winkler, M. E., & Blaber, M. (1986) *Biochemistry 25*, 4041−4045.

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