Analysis of the Forces Which Stabilize the Active Conformation of Urokinase-Type Plasminogen Activator†

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ABSTRACT: It was recently proposed that hydrophobic interactions control the active conformation of serine proteases in the trypsin family (Hedstrom et al. (1996) *Biochemistry 35*, 4515−23) rather than a charge interaction with Asp next to the active site Ser, as formerly believed. In the present study, certain site-directed mutants of the serine protease zymogen pro-urokinase (pro-UK) and its two-chain enzymatic derivative urokinase (UK) were characterized. The results provide information on the structure−function of the catalytic domain of pro-UK/UK, which is relevant to this controversy. Mutations at $Asp^{355(c194)}$, which eliminated its charge, induced a 6250-fold reduction in the catalytic activity of UK. By contrast, reducing the hydrophobicity at the neoterminal $Ile^{159(c16)}$ of UK had relatively little effect. However, when both the hydrophobicity and the size of the side chain were reduced by a glycine substitution at this position, a major reduction (9090-fold) in the catalytic efficiency of UK occurred. This effect was related to the smaller side chain increasing the cavity and the flexibility of the N-terminus and thereby interfering with its charge interaction with $Asp^{355(c194)}$. A similar mechanism, rather than a change in hydrophobicity, is believed also to explain the reduction in the stabilization energy of the activation domain observed in a trypsin mutant by Hedstrom et al. (1996). Although hydrophobic interaction facilitated the charge interaction with $Asp^{355(c194)}$, the latter was the primary force which stabilized the active conformation of UK. The charge interaction with $Asp^{355(c194)}$ was also found to be the principal determinant of the intrinsic catalytic activity of single-chain pro-UK. Additionally, the findings confirmed that the K_M of pro-UK for its natural substrate was significantly lower than that of UK. Since this same phenomenon was also seen with each of the mutants, the substrate binding pocket of these single-chain zymogens was better formed than that of their two-chain, enzymatic derivatives.

A typical serine protease zymogen is activated by proteolytic cleavage, which releases a neoterminal hydrophobic residue, such as Ile or Val. The neoterminal hydrophobic residue then forms a salt bridge with the Asp next to the active site Ser, which stabilizes the active conformation (*1*−*3*). This is an example of protein folding driven by electrostatic interactions rather than by hydrophobic forces, which are generally considered to be the primary forces involved in folding and stabilizing proteins (*4*,*5*).

Since the energy required to stabilize the active conformation is about 8.0 kcal/mol (*6*,*7*) this concept of protease activation was contradicted by the finding that the salt bridge of Asp^{194}−Ile^{16} contributes only 2.9 kcal/mol to stabilize the active conformation of δ-chymotrypsin, based on the measurement of pH shift (*8*). Recently, using trypsin mutants, Hedstrom et al. (*9*) showed that disrupting the salt bridge resulted in only a $\Delta\Delta G^{\ddagger}$ of <3.4 kcal/mol, whereas disrupting hydrophobic interactions generated by the side chain of Ile^{16} was found to account for a $\Delta\Delta G^{\ddagger}$ of <5.7 kcal/mol. They concluded that hydrophobic interactions are more critical for stabilizing the active conformations of serine proteases. They further suggested these hydrophobic interactions may also be important in "active" zymogens, including pro-urokinase (pro-UK).[1] Since pro-UK and its enzyme, urokinase (UK), were found to have some unique catalytic properties (*10*−*22*), certain mutants of pro-UK were made and studied to test whether this new theory was applicable to pro-UK/UK.

Pro-UK/UK belongs to the trypsin family of serine proteases. It has a very specific proteolytic activity against a single peptide bond (Arg^{560}−Val^{561}) in plasminogen. Like other serine protease zymogens, pro-UK is activated to UK after cleavage of a single peptide bond ($Lys^{158(c15)}$−$Ile^{159(c16)}$) by certain proteases such as plasmin, kallikrein, and cathepsin B. However, unlike the cases for most other serine protease zymogens, activation of pro-UK results in only a 500−1000-fold increase in activity (*12*−*14*,*16*−*18*), which is equivalent to only a $\Delta\Delta G^{\ddagger}$ of 3.7−4.1 kcal/mol, in contrast to a $\Delta\Delta G^{\ddagger}$

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[1] Abbreviations: $^{(cnnn)}$, chymotrysin numbering (nnn); pro-UK,single chain u-PA; UK, urokinase or two-chain u-PA; t-PA, tissue plasminogen activator; DFP, diisopropylfluorophosphate; A, absorbance.

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of 8.0 kcal/mol for most other serine proteases. This is because pro-UK has a relatively high intrinsic catalytic activity, which is about 1000−2000-fold higher than that of trypsinogen. A positively charged residue, $Lys^{300(c143)}$, acting as a surrogate for the neo-N-terminal isoleucine, was identified to be primarily responsible for this unusual intrinsic activity (*23*). Unlike the single-chain activity of t-PA, the intrinsic activity of pro-UK was found to be unaffected by mutating $Lys^{313(c156)}$ to Ala (*24*) and was only slightly (2−fold) reduced by restoring the zymogenic triad (*25*). By contrast, the high activity of single-chain t-PA has been related mostly to a loss of the zymogenic triad (*26*) and to several individual residues such as $Lys^{416(c143)}$ (*27*), $His^{417(c144)}$ (*28*), and $Lys^{429(c156)}$ (*29*−*31*).

EXPERIMENTAL PROCEDURES

Materials. The synthetic chromogenic substrate for UK (S2444, L-pyroglutamyl-glycyl-L-arginine-*p*-nitroanilide hydrochloride) was obtained from Kabi (Franklin, OH). Lys-plasmin was obtained from American Diagnostica (Greenwich, CT). Enzymes and reagents for gene manipulation, expression, and site-directed mutagenesis 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) Gene Construction, Expression, and Purification of Pro-UK and Its Mutants. The gene for native pro-UK has been well characterized (*32*), and its cDNA was obtained from Dr. Paolo Sarmientos (Farmitalia, Milano, Italy). The site-directed mutants ($Ile^{159(c16)}$→Val, $Ile^{159(c16)}$→Ala, $Ile^{159(c16)}$→Gly, $Asp^{355(c194)}$→Asn & $Asp^{355(c194)}$→Ala) of pro-UK were constructed and expressed in *Escherichia coli* as follows: The cDNA of the pro-UK mutant was obtained by site-directed mutagenesis after subcloning the HindIII−Bam HI restriction fragment from a pFC16 plasmid containing the full-length cDNA of pro-UK (*33*) into an M13 vector (mp18). A specific synthetic oligonucleotide coding for the designed site mutant was first hybridized to the recombinant M13 construct and then extended with T4 DNA polymerase. Following ligation and transformation, positive clones were selected for DNA sequencing. The expression plasmid for the mutant was constructed by reinserting the mutated HindIII−BamHI fragment in pFC16 and introduced into an *E. coli* type B strain. The pro-UK mutants were purified from sonicated cell lysates by chromatography through an S-Sepharose, pro-UK antibody affinity column and Sephadex G-25 after refolding using the method previously described (*34*). Purified mutants were observed as single bands on reduced and nonreduced SDS−PAGEs with silver stain. Protein concentration was determined from absorbance at 280 nm using the extinction coefficient ($E^{1\%}_{280\ nm} = 13.6$) of pro-UK. Usually, the folded pro-UK proteins are active and soluble, and the inactive molecules are misfolded/unfolded and insoluble (unpublished observation). Therefore, the misfolded proteins were unlikely to be included in the purified samples.

(2) Conversion of Pro-UK or Its Single-Chain Form Mutants (Pro-UK Mutants) to UK or Two-Chain Form Mutants (UK Mutants) by Lys-plasmin. Pro-UK or mutants (10 μM) were incubated with 40 nM Lys-plasmin at 37 °C for 90 min. To stop the reaction and remove added Lys-plasmin, 50 μL of aprotinin-Sepharose was added to each reaction mixture and was incubated at 37 °C for 30 min and then was removed by centrifugation. Complete removal of plasmin was ensured by assay with the synthetic substrate S2251 (1.5 mM). The conversion to two-chain forms (UK/mutants) was confirmed by reduced SDS−PAGE.

(3) Intrinsic Catalytic Activity of Pro-UK or Pro-UK Mutants against S2444. Pro-UK (4 μM) or pro-UK mutants {$Ile^{159(c16)}$→Val (4 μM), $Ile^{159(c16)}$→Ala (10 μM), $Ile^{159(c16)}$→Gly (10 μM), $Asp^{355(c194)}$→Asn (10 μM), or $Asp^{355(c194)}$→Ala (10 μM)} were incubated with a range of concentrations (0−24 mM) of S2444 in 0.05 M Tris-HCl, 0.1 M NaCl, 0.1% BSA, and 0.01% tween 80, pH 7.4, at room temperature. The buffer along with S2444 was used as a control. The reaction rate was measured by the linear optical density (OD) increase with time at 410 nm against a reference wavelength of 490 nm on a microtiter plate reader. The kinetic analysis was performed as previously described (*17*).

(4) Activity of UK or UK Mutants against S2444. The activity of UK (0.1 nM) or UK mutants {$Ile^{159(c16)}$→Val (0.1 nM), $Ile^{159(c16)}$→Ala (5 nM), $Ile^{159(c16)}$→Gly (1 μM), $Asp^{355(c194)}$→Asn (1 μM), or $Asp^{355(c194)}$→Ala (5 μM)} was assayed with S2444, as described above. The effect of pH on the reactions was also studied for the wild-type UK and the two-chain $Asp^{355(c194)}$→Asn mutant by varying the pH (*6*−*10*) of the reaction mixture.

RESULTS AND DISCUSSION

The Charge Interaction between $Ile^{159(c16)}$ and $Asp^{355(c194)}$ Stabilized the Activation Conformation of UK (Table 1). Unlike the trypsin mutant Asp^{194}→Asn, which only induced a modest reduction (145-fold) in its activity ($\approx\Delta\Delta G^{\ddagger}$ of 3.0 kcal/mol) against synthetic substrates (*9*), the UK mutant $Asp^{355(c194)}$→Asn (at pH 7.4) induced a 6250-fold reduction ($\Delta\Delta G^{\ddagger}$, 5.2 kcal/mol) in catalytic efficiency (k_{cat}/K_M) against the synthetic substrate S2444 (Table 1A). This was due to an 18-fold increase in K_M and a 360-fold decrease in k_{cat}. In contrast, the trypsin mutant Asp^{194}→Asn induced a 29-fold increase in K_M and only a 5-fold decrease in k_{cat} against the substrate Tos-Gly-Pro-Arg-AMC.

To eliminate the effect of protonation of the free α-amino-group of $Ile^{159(c16)}$, the reaction was studied at high pH (*8*−*10*), since the pK_a for the free amino-terminal is about 8 (8). The $\Delta\Delta G^{\ddagger}$ was maximally changed (0.4 kcal/mol) with the $Asp^{355(c194)}$→Asn mutant over the pH range 7.4−10.0 (Table 1B), probably due to the penalty for burying the protonated N-terminus. The $\Delta\Delta G^{\ddagger}$ was also changed up to 0.3 kcal/mol for the wild-type UK at this same pH range (7.4−10.0), probably due to further deprotonation of the active site $His^{215(c57)}$. The $\Delta\Delta G^{\ddagger}$ between UK and the mutant remained unchanged (5.0−5.2 kcal/mol) over this pH range. This excluded the possibility that the protonated N-terminus of the $Asp^{355(c194)}$→Asn mutant contributed significantly to the loss (5 kcal/mol) of the stabilizing energy of the active conformation.

The loss of the stabilizing energy (5.2 kcal/mol) may also include contributions from other forces, such as hydrophobic interactions with $Ile^{159(c16)}$ and a cavity partially filled with the side chain of $Lys^{300(c143)}$, which were induced or enhanced

Table 1: Kinetic Analysis of the Hydrolysis of S2444 (<Glu-Gly-Arg-pNA HCl) by UK and Mutants[a]

A	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (μM^{-1} min^{-1})	ratio (fold) (UK/mutants)	$\Delta\Delta G^{\ddagger}$ (kcal/mol)
UK	86 ± 16	161 ± 17	1.87	1	0
$Asp^{355(c194)}$→Asn	1540 ± 320	0.45 ± 0.14	2.93×10^{-4}	6250	5.2
$Asp^{355(c194)}$→Ala	12700 ± 3100	0.33 ± 0.12	2.61×10^{-5}	71430	6.6
$Ile^{159(c16)}$→Val	96 ± 20	167 ± 19	1.74	1.07	0.043
$Ile^{159(c16)}$→Ala	880 ± 170	82 ± 14	9.35×10^{-2}	20	1.8
$Ile^{159(c16)}$→Gly	7950 ± 950	1.62 ± 0.8	2.03×10^{-4}	9090	5.4

B	Wild-Type UK							
pH	6.0	6.7	7.4	8.0	8.5	9.0	9.5	10.0
k_{cat}/K_M (μM^{-1} min^{-1})	0.16	0.58	1.87	2.24	2.95	2.74	2.62	2.17
ratio (fold)	0.09	0.31	1	1.20	1.58	1.47	1.40	1.16
$\Delta\Delta G^{\ddagger}$ (kcal/mol)	−1.42	−0.69	0	0.11	0.27	0.23	0.20	0.09
	$Asp^{355(c194)}$→Asn							
pH	6.0	6.7	7.4	8.0	8.5	9.0	9.5	10.0
k_{cat}/K_M (μM^{-1} min^{-1})	0.11	0.65	2.93	3.85	5.31	5.86	5.80	5.45
ratio (fold)	0.04	0.22	1	1.31	1.81	2.00	1.98	1.86
$\Delta\Delta G^{\ddagger}$ (kcal/mol)	−1.95	−0.89	0	0.16	0.35	0.41	0.40	0.37

[a] Michaelis−Menten parameters are listed for the hydrolysis of S2444 by wild-type and mutant UK. Values reported are the average of at least three independent experiments (± SEM for part B). Assays were performed in 50 mM Tris-HCl, 100 mM NaCl, 0.01% Tween 80, at 25 °C, as described under Experimental Procedure (with pH 7.4 for part A and varied pH for part B). $\Delta\Delta G^{\ddagger}$ is calculated from the ratio of k_{cat}/K_M.

Table 2: Kinetic Analysis of the Hydrolysis of S2444 (<Glu-Gly-Arg-pNA HCl) by Pro-UK and Mutants[a]

	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (μM^{-1} min^{-1})	ratio (fold) (pro-UK/ mutants)	$\Delta\Delta G^{\ddagger}$ (kcal/mol)
pro-UK	56 ± 6	0.1 ± 0.2	1.78×10^{-3}	1	0
$Asp^{355(c194)}$→Asn	1250 ± 180	$(8.3 \pm 1.6) \times 10^{-3}$	6.6×10^{-6}	270	3.3
$Asp^{355(c194)}$→Ala	5500 ± 1200	$(6.0 \pm 0.8) \times 10^{-3}$	1.1×10^{-6}	1630	4.4
$Ile^{159(c16)}$→Val	85 ± 10	0.22 ± 0.05	2.59×10^{-3}	0.7	−0.2
$Ile^{159(c16)}$→Ala	157 ± 27	$(7.0 \pm 1.9) \times 10^{-2}$	4.5×10^{-4}	4	0.8
$Ile^{159(c16)}$→Gly	4720 ± 980	$(3.9 \pm 0.7) \times 10^{-2}$	8.4×10^{-6}	213	3.2

[a] Michaelis−Menten parameters are listed for the hydrolysis of S2444 by wild-type and mutant pro-UK. Values reported are the average of at least three independent experiments ± SEM. Assays were preformed in 50 mM Tris-HCl, 100 mM NaCl, 0.01% Tween 80, pH 7.4, at 25 °C, as described under Experimental Procedure. $\Delta\Delta G^{\ddagger}$ is calculated from the ratio of k_{cat}/K_M for a mutant and the wild-type.

by the charge interactions with $Asp^{355(c194)}$. The stabilizing energy for filling the cavity with the side chain of $Lys^{300(c143)}$ can be estimated to be less than 0.6 kcal/mol, on the basis of previous finding that the UK mutant $Lys^{300(c143)}$→Ala reduced the catalytic efficiency by 3-fold (*23*). The contribution from hydrophobic interactions with $Ile^{159(c16)}$ enhanced by the charge interaction with $Asp^{355(c194)}$ is difficult to estimate and should be considered as a consequence of the salt bridge. These interactions were probably absent in trypsin, since the absence of the charge at 194 only resulted in a 3 kcal/mol loss of energy.

Therefore, these data indicated that eliminating the charge at this residue had a major effect on the catalytic activity of UK, reflecting the importance of the charge interaction between $Ile^{159(c16)}$ and $Asp^{355(c194)}$ for stabilizing the activation domain of UK.

This interpretation of the findings was verified by another UK mutant, $Asp^{355(c194)}$→Ala, in which not only the charge was eliminated but also the size of the side chain was reduced. This mutation induced a further reduction in catalytic efficiency to 71430-fold ($\Delta\Delta G^{\ddagger}$ of 6.6 kcal/mol), related to a further increase in K_M to 12700 μM, which compares with 86 μM for UK and 1540 μM for the mutant $Asp^{355(c194)}$→Asn. These data suggest that the side chain of $Asp^{355(c194)}$ may be involved in the formation of the substrate binding pocket of UK. It is also suggested that the cavity brought by the smaller side chain of alanine further decreases the stability of the active conformation.

Hydrophobic Interactions Facilitated the Charge Interaction between $Ile^{159(c16)}$ and $Asp^{355(c194)}$ (Table 1A). To investigate the role of the hydrophobicity of $Ile^{159(c16)}$ in the formation of the active conformation of UK, several UK mutations were made to gradually reduce hydrophobicity at $Ile^{159(c16)}$. The Eisenberg's hydrophobic index (EHI) (*35*) for isoleucine is 1.38. As was found with the trypsin mutant Ile^{16}→Val ($\Delta\Delta G^{\ddagger}$ of −0.3 kcal/mol) (*9*), little change ($\Delta\Delta G^{\ddagger}$ of 0.043 kcal/mol) in catalytic efficiency was induced in UK by mutating $Ile^{159(c16)}$ to valine, which is slightly less hydrophobic (EHI = 1.08) and has a one-methylene-shorter side chain. When the substitution was alanine, which is much less hydrophobic (EHI = 0.62) and has only one methyl group as the side chain, the catalytic efficiency of UK was reduced by 20-fold ($\Delta\Delta G^{\ddagger}$, 1.8 kcal/mol). The comparable trypsin mutant Ile^{16}→Ala was reported to induce an 83-fold reduction ($\Delta\Delta G^{\ddagger}$, 2.6 kcal/mol) in catalytic efficiency (*9*), indicating that the hydrophobic effect of $Ile^{159(c16)}$ was somewhat less influential in UK than in trypsin. Since it was found that mutating $Lys^{300(c143)}$ to alanine reduced the two-chain activity of UK by 3-fold (*23*), one possible explanation is that $Lys^{300(c143)}$, which is absent in

Table 3: Ratio of Catalytic Efficiencies (k_{cat}/K_M) between UK Mutants and Pro-UK Mutants

	k_{cat}/K_M (μM^{-1} min^{-1})			
	single-chain	two-chain	ratio (fold) (tc/sc)	$\Delta\Delta G^{\ddagger}$ (kcal/mol)
wild-type u-PA	1.78×10^{-3}	1.87	1050	4.1
$Asp^{355(c194)} \rightarrow Asn$	6.6×10^{-6}	2.93×10^{-4}	44	2.2
$Asp^{355(c194)} \rightarrow Ala$	1.1×10^{-6}	2.61×10^{-5}	24	1.9
$Ile^{159(c16)} \rightarrow Val$	2.59×10^{-3}	1.74	672	3.8
$Ile^{159(c16)} \rightarrow Ala$	4.5×10^{-4}	9.35×10^{-2}	210	3.2
$Ile^{159(c16)} \rightarrow Gly$	8.4×10^{-6}	2.03×10^{-4}	24	1.9

trypsin, may somehow compensate for the loss of stability caused by mutating $Ile^{159(c16)}$ to alanine. This would be verified by a $Ile^{159(c16)}/Lys^{300(c143)} \rightarrow Ala$ double mutation. The possible compensation from its single-chain active conformation was negligible, since the two-chain activity of the mutant $Ile^{159(c16)} \rightarrow Ala$ was much higher (53- and 210-fold, respectively) than the single-chain activity of the wild-type pro-UK or the $Ile^{159(c16)} \rightarrow Ala$ mutant (Table 3).

Finally, when the substitution was glycine, which has only a hydrogen atom as its side chain, a major reduction (9090-fold and $\Delta\Delta G^{\ddagger}$ of 5.4 kcal/mol) in the catalytic efficiency of UK was induced. Since the hydrophobicity of glycine (EHI = 0.48) is comparable to that of alanine (EHI = 0.62), it is probably insufficient to explain the predominant effect of the glycine substitution (9090 vs 20-fold). Two other factors were additionally considered. First, the cavity generated by the smaller side chain of glycine could be responsible for the instabilibity. Second, the glycine substitution could impair the charge interaction with $Asp^{355(c194)}$, because its essentially absent side chain made the N-terminus too flexible for the charge interaction. A major reduction (14925-fold and $\Delta\Delta G^{\ddagger}$ of 5.7 kcal/mol) in catalytic efficiency of trypsin was also reported for a comparable trypsin mutant, $Ile^{16} \rightarrow Gly$, and was attributed to the hydrophobic force hypothesis by Hedstrom et al. (*9*). However, as with the UK mutant, this glycine substitution probably also affected the cavity and the charge interaction between Asp^{194} and Ile^{16}, which were not considered as possibilities. Although hydrophobic interactions were somewhat more influential in trypsin (83-fold) than in UK (20-fold), as evidenced by the $Ile^{159/16} \rightarrow Ala$ mutations, the effect of the glycine substitution does not adequately support that hydrophobic force hypothesis. Nevertheless, it is reasonable to postulate that the initial positioning of the N-terminus ($Ile^{159/16}$) is absolutely dependent on the hydrophobic interactions, which is critical for formation of the salt bridge. Furthermore, the hydrophobic interactions probably also stabilize the salt bridge by reducing the mobility of the N-terminus.

In conclusion, these data indicate that the hydrophobic interactions in both UK and trypsin facilitate the charge interaction between $Ile^{16/159}$ and $Asp^{194/355}$, but at least in UK, it is the charge interaction which is primarily responsible for stabilizing the active conformation.

The Intrinsic Catalytic Activity of Pro-UK Was Also Dependent on a Charge Interaction with $Asp^{355(c194)}$ (Table 2). The intrinsic catalytic activity of pro-UK was reduced 270 and 1630-fold ($\Delta\Delta G^{\ddagger}$ of 3.3 and 4.4 kcal/mol) by the pro-UK mutants $Asp^{355(c194)} \rightarrow Asn$ and $Asp^{355(c194)} \rightarrow Ala$, respectively, which also significantly reduced the activity of UK by eliminating the charge at this residue. Therefore, the intrinsic catalytic activity of pro-UK was similarly dependent on a charge interaction with $Asp^{355(c194)}$ for its "active" conformation. However, in the case of the single-chain protease pro-UK, the residue which interacted with $Asp^{355(c194)}$ was shown to be a positively charged internal residue, $Lys^{300(c143)}$, instead of the neo-N-terminal isoleucine$^{159(c16)}$ in the two-chain protease. This charge interaction was primarily responsible for the intrinsic catalytic activity of pro-UK (*23*). As with the UK mutants, the $Asp^{355(c194)}$ mutants induced a large increase in the K_M of pro-UK (1250 μM for $Asp^{355(c194)} \rightarrow Asn$ and 5500 μM for $Asp^{355(c194)} \rightarrow Ala$), suggesting that the side chain of $Asp^{355(c194)}$ was similarly involved in the formation of the substrate binding pocket of pro-UK (K_M, 56 μM).

The pro-UK mutants ($Ile^{159(c16)} \rightarrow Val$ and $Ile^{159(c16)} \rightarrow Ala$) which reduced the hydrophobicity at $Ile^{159(c16)}$, had no significant effect on the intrinsic catalytic activity. However, the mutant $Ile^{159(c16)} \rightarrow Gly$ reduced the intrinsic catalytic activity by 213-fold ($\Delta\Delta G^{\ddagger}$, 3.2 kcal/mol) for reasons which are unclear. However, according to the 3-d structural model of pro-UK (*23*), $Ile^{159(c16)}$ is located in a peptide (149−163) spatially close to $Lys^{300(c143)}$. The glycine substitution could increase the mobility of the peptide (149−163), and thereby interfere with the charge interaction between $Lys^{300(c143)}$ and $Asp^{355(c194)}$.

It is unlikely that the effect of pro-UK mutants on the intrinsic catalytic activity was due to contaminants of two-chain forms or other proteases in the samples. First, certain other mutants produced and prepared using the same system were found to have higher two-chain activity but reduced intrinsic catalytic activity ($Lys^{300(c143)} \rightarrow His$) or to have lower two-chain activity but higher intrinsic catalytic activity ($Glu^{301(c144)} \rightarrow His$) (*24*). This excluded the above possibility of contamination. Additionally, purified mutants including the mutant $Ile^{159(c16)} \rightarrow Gly$ were observed as single bands on both reduced and nonreduced SDS−PAGEs with silver stain, as described in the Experimental Procedure.

Activation of the Pro-UK Mutants Suggests That Serine Proteases May Be Catalytically Active Even in the Absence of $Asp^{355(c194)}$ (Table 3). The changes in Gibbs free energy ($\Delta\Delta G^{\ddagger}$) during the activation of the pro-UK mutants were reduced from 4.1 kcal/mol for wild-type pro-UK to 3.8, 3.2, 1.9, 2.2, and 1.9 kcal/mol for the mutants $Ile^{159(c16)} \rightarrow Val$, $Ile^{159(c16)} \rightarrow Ala$, $Ile^{159(c16)} \rightarrow Gly$, $Asp^{355(c194)} \rightarrow Asn$, and $Asp^{355(c194)} \rightarrow Ala$, respectively. This reduction was due to a loss of two-chain enzyme activity by the mutations. However, there remained a residual $\Delta\Delta G^{\ddagger}$ of 1.9−2.2 kcal/mol for the activation of the pro-UK mutants $Ile^{159(c16)} \rightarrow Gly$, $Asp^{355(c194)} \rightarrow Asn$, and $Asp^{355(c194)} \rightarrow Ala$. This suggested that a conformation change occurred in the molecule during the conversion from a single-chain to a two-chain form, which induced a catalytic active-site-like conformation even in the absence of the major stabilizing forces for the active site.

The "Real" Zymogenic State of Pro-UK (Figure 1). Unlike other typical serine protease zymogens, pro-UK has an active conformation while it remains single chain. This active conformation is much less stable ($\Delta\Delta G^{\ddagger}$ of 4.1 kcal/mol) than its other active conformation, which occurs when it is converted to the two-chain form. The $Asp^{355(c194)} \rightarrow Asn$ mutant disrupts the charge interaction between $Asp^{355(c194)}$

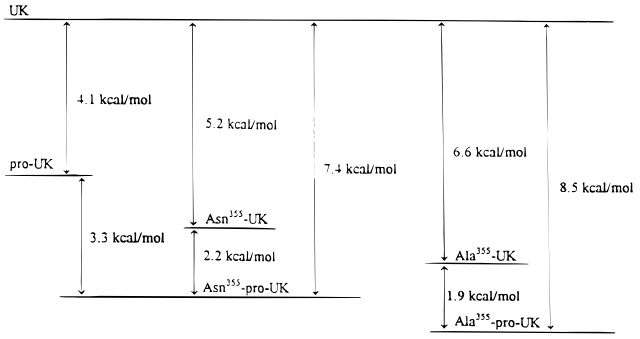

FIGURE 1: Summary of the loss of the stabilizing energy for active conformations of pro-UK/UK by mutants, calculated based on the ratio of k_{cat}/K_M for mutants and the wild-type.

Table 4: K_M of u-PA and Mutants

	K_M (μM)	
	single-chain	two-chain
wild-type u-PA	56	86
$Asp^{355(c194)}\rightarrow Asn$	1250	1540
$Asp^{355(c194)}\rightarrow Ala$	5500	12700
$Ile^{159(c16)}\rightarrow Val$	85	96
$Ile^{159(c16)}\rightarrow Ala$	157	880
$Ile^{159(c16)}\rightarrow Gly$	4720	7950

and $Lys^{300(c143)}$, the primary stabilizing force for the active conformation of single-chain pro-UK, and brought the pro-UK to its "real" zymogenic state. There is a difference of 7.4 kcal/mol in $\Delta\Delta G^{\ddagger}$ between the pro-UK mutant $Asp^{355(c194)}\rightarrow Asn$ and the wild-type UK, which is close to those between other zymogens and serine proteases. A further reduction of $\Delta\Delta G^{\ddagger}$ (8.5 kcal/mol) was found with the pro-UK mutant $Asp^{355(c194)}\rightarrow Ala$. The loss of 1.1 kcal/mol was probably due to the cavity of $Asp^{355(c194)}\rightarrow Ala$.

Although both pro-UK and t-PA have an active conformation in the single-chain form, they are quantitatively and functionally very different. The single-chain activity of t-PA is only 4−7-fold less than its two-chain activity (*26*,*27*,*36*), which makes single-chain t-PA an enzyme which reacts with serpins in plasma (*37*). In contrast, pro-UK has an intrinsic catalytic activity which is 0.1−0.4% of its two-chain activity (*12*−*14*,*17*), and is inert in plasma (*11*). It has also been shown that the structural basis for their single-chain activities is very different, although they are the most homologous members of the serine protease family (*23*−*25*).

The Substrate Binding Pockets of Pro-UK and All the Pro-UK Mutants Were Better Formed Than Their Respective Two-Chain Enzyme Forms (Table 4). It was noteworthy that the K_M values of pro-UK and all its mutants were consistently smaller than those of their two-chain counterparts. This suggested that the substrate binding pockets of the single-chain forms were better formed than those of the two-chain enzymes. It has been previously observed that the K_M of pro-UK is significantly smaller than that of UK against its native substrate, glu-plasminogen (*10*,*16*,*17*). The functional importance of this low K_M was previously demonstrated by the findings that pro-UK could be fully active against fibrin or cell-bound plasminogen (*16*,*17*,*19*,*21*,*22*) and that a hyperactive transitional state against plasminogen was found during the conversion of pro-UK to UK (*15*). However, an alternative explanation that nonproductive substrate binding due to a deformed substrate binding site will cause a decrease in K_M is not currently excluded.

In conclusion, the data from these UK mutants do not support the hydrophobic force theory of Hedstrom et al. (*9*), at least not for the activation of this serine protease zymogen. Instead, the charge interaction with Asp^{355} was found to be the primary force which stabilizes the activation conformation of UK and the intrinsic catalytic activity of pro-UK.

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