Deletion of Ile1 Changes the Mechanism of Streptokinase: Evidence for the Molecular Sexuality Hypothesis†

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ABSTRACT: Plasminogen (Plgn) is usually activated by proteolytic cleavage of Arg561−Val562. The new N-terminal amino group of Val562 forms a salt bridge with Asp740, creating the active protease plasmin (Pm). However, streptokinase (SK) binds to Plgn, generating an active protease in a poorly understood, nonproteolytic process. We hypothesized that the N-terminus of SK, Ile1, substitutes for the N-terminal Val562 of Pm, forming an analogous salt bridge with Asp740. SK initially forms an inactive complex with Plgn, which subsequently rearranges to create an active complex; this rearrangement is rate limiting at 4 °C. SK·Plgn efficiently hydrolyzes amide substrates at 4 °C, although ΔIle1−SK·Plgn has no amidolytic activity. ΔIle1−SK prevents formation of wild-type SK·Plgn. These results indicate that ΔIle1−SK forms the initial inactive complex with plasminogen, but cannot form the active complex. However, when the experiment is performed at 37 °C, amidolytic activity is observed when ΔIle1−SK is added to plasminogen. SDS−PAGE analysis demonstrates that the amidolytic activity results from the formation of ΔIle1−SK·Pm. To further demonstrate that the activity of ΔIle1−SK requires the conversion of Plgn to Pm, we characterized the reaction of SK with a mutant microplasminogen, Arg561Ala−μPlgn, that cannot be converted to microplasmin. Amidolytic activity is observed when Arg561Ala−μPlgn is incubated with wild-type SK at 37 °C; however, no amidolytic activity is observed in the presence of ΔIle1−SK. These observations demonstrate that the amidolytic activity of ΔIle1−SK at 37 °C requires the conversion of Plgn to Pm. Our findings indicate that Ile1 of SK is required for the nonproteolytic activation of Plgn by SK and are consistent with the hypothesis that Ile1 of SK substitutes for Val562 of Pm.

Like the blood coagulation pathway, the fibrinolytic pathway involves the sequential activation of trypsin-like serine proteases. The conversion of plasminogen to plasmin is the final step in the fibrinolytic pathway; plasmin is the protease responsible for the lysis of blood clots (*1*, *2*). This conversion involves proteolysis at the Arg561−Val562 bond of plasminogen; the new N-terminal amino group is believed to form a salt bridge with the carboxylate of Asp740 in a manner analogous to the activation of trypsinogen to trypsin (*3*) (chymotrypsinogen numbering, Table 1). Plasminogen activators catalyze the conversion of plasminogen to plasmin and are important chemotherapeutic agents in the treatment of myocardial infarction and ischemic stroke. u-PA[1] and t-PA are the proteases that catalyze the activation of plasminogen in man. SK and staphylokinase are bacterial-derived plasminogen activators. Unlike u-PA and t-PA, SK and staphylokinase are not proteases (nor do they have "kinase" activity). Thus, both SK and staphylokinase must recruit protease activity from another protein. SK can form a plasminogen activator by two pathways (Figure 1). Pathway 1: SK forms a complex with plasminogen (SK·Plgn). This initial complex rearranges to form an active complex (SK·Plgn*) which has approximately the same amidolytic activity as plasmin (*4*). Thus the binding of SK to plasminogen induces formation of a functional active site in plasminogen (*5*, *6*). Moreover, SK·Plgn* can catalyze the conversion of additional plasminogen molecules to plasmin, i.e., SK·Plgn* is a plasminogen activator. The rearrangement of SK·Plgn to SK·Plgn* is rate limiting at low temperature (*5*, *7*). SK·Plgn* may undergo further rearrangements to form additional

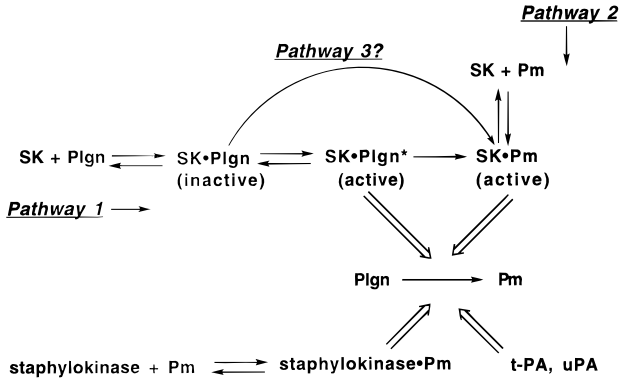

FIGURE 1: Mechanism of SK and staphylokinase activation of plasminogen.

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[1] Abbreviations: SK, streptokinase; Pm, plasmin; Plgn, plasminogen; PA, plasminogen activator; u-PA, urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate; μPlgn, microplasminogen; μPm, microplasmin; DTT, dithiothreitol; GdnHCl, guanidine hydrocholoride.

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active complexes and is subsequently hydrolyzed to produce the SK-plasmin complex (SK·Pm) (*7*). Although plasmin cannot activate plasminogen, SK·Pm is also a plasminogen activator (*8*). Thus, SK also changes the specificity of plasmin. Pathway 2: SK can form a plasminogen activator by complexing directly with plasmin. Trace amounts of plasmin are always present in plasminogen preparations, and plasmin appears to have a higher affinity for SK (*9*). In contrast to SK, the activation of plasminogen by staphylokinase appears to follow only the second pathway. Unlike SK·Plgn*, the staphylokinase−plasminogen complex is inactive. Instead, staphylokinase combines with plasmin; this staphylokinase−plasmin complex catalyzes the activation of plasminogen (*10*, *11*). The difference in the mechanisms of SK and staphylokinase is illustrated by their reaction with Arg561Ala-plasminogen. This plasminogen contains a mutation which prevents conversion to plasmin. The complex between Arg561Ala−plasminogen and SK has amidolytic activity, which indicates that SK·Plgn* forms. However, the complex with staphylokinase complex is inactive, which indicates that plasminogen activation by staphylokinase requires the formation of plasmin (*11*).

To the first approximation, the process of zymogen activation is identical among the enzymes of the trypsin family (*12*). Therefore, it is reasonable to expect that the activation of plasminogen involves rearrangement of the four peptide segments analogous to the activation domain of trypsinogen. This conformational change is triggered either by the proteolytic release of Val562 in plasmin or by the binding of SK. Interestingly, the N-terminus of SK is Ile1−Ala2−Gly3, which is very similar to the N-termini of plasmin (Val562−Val563−Gly564) and trypsin (Ile16−Val17−Gly18). Bode and Huber have proposed that the N-terminus of SK forms a salt bridge with Asp740 of plasminogen, triggering the conformational change in a manner analogous to the N-terminal Val562 of plasmin (*13*). They termed this mechanism "molecular sexuality".

While this proposal has never been explicitly tested, several pieces of evidence appear to argue against the molecular sexuality hypothesis. Several laboratories have reported that the removal of the first 59 residues of SK impairs, but does not abolish, activity (*14*, *15*). No activity is lost when the N-terminus of SK is blocked by fusion proteins or truncated by 16 residues (*16*−*18*). Unfortunately, none of these experiments were performed under conditions where the conformational rearrangement is rate limiting and where trace amounts of plasmin are not present. Since SK has a higher affinity for plasmin than for plasminogen (*9*), these experiments may measure the effects of the mutations on SK·Pm rather than on SK·Plgn*. Last, truncation of SK may well impair formation of SK·Plgn* by pathway 1, but leave pathway 2 intact—in effect changing the mechanism of SK into that of staphylokinase. Such a change in mechanism could easily be overlooked.

We decided to test the molecular sexuality hypothesis by deleting Ile1 from SK. This mutation abolishes the activation of plasiminogen by SK under conditions where the conversion of SK·Plgn to SK·Plgn* is rate limiting. These experiments demonstrate the importance of the N-terminus of SK in plasminogen activation and provide compelling evidence for the molecular sexuality hypothesis.

MATERIALS AND METHODS

Materials. D-Val-Leu-Lys-pNA and plasmin were purchased from Sigma Chemical Co. Human plasminogen was isolated from fresh frozen plasma following known procedures and pretreated with soybean trypsin inhibitor resin to remove contaminating plasmin (*19*). Factor Xa was purchased from New England Biolabs. Human Glu-plasminogen was purchased from Enzyme Research (South Bend, IN).

Construction, Expression, and Purification of ΔIle1−SK. Wild-type and ΔIle1−SK were produced as fusion proteins with maltose binding protein using the pMal-c vector (New England BioLabs) (*16*). The ΔIle1 mutation was introduced using a QuikChange Site Directed Mutagenesis Kit (Stratagene). The protein was expressed in XL1-Blue cells (Stratagene). A 10 mL overnight culture was diluted into 1 L of LB broth containing 100 μg/mL ampicillin. Protein expression was induced when the optical density reached 0.6−0.8 by addition of 0.4 mM IPTG. The cells were harvested after 6 h. The cell pellet was resuspended in 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5% sucrose and lysed by sonication at 4 °C. The cell debris was removed by centrifugation at 19 000 rpm (JA20 rotor) for 25 min. The supernatant was applied to an amylose column preequilibrated in 20 mM Tris-HCl, pH 7.4, and 2 mM EDTA. The fusion protein was eluted in 10 mM maltose, 20 mM Tris-HCl, pH 7.4, and 2 mM EDTA. The SK was separated from the MBP by treatment with Factor Xa for 24 h at room temperature. SK was purified on an HQ anion-exchange column (PerSeptive Biosystems) using a linear gradient of 25 mM to 500 mM NaCl in 25 mM Tris-HCl, pH 8.0. The protein was concentrated and stored in 50% glycerol at −20 °C. The N-termini were confirmed by N-terminal sequencing at the Tufts Medical School Protein Sequencing Facility.

Expression and Purification of Microplasminogen. A plasminogen clone was provided by Dr. Francis Castellino (University of Notre Dame, IN). The microplasminogen coding sequence [residues 530−791, microplasminogen as defined by Shi and Wu (*20*), not Wang et al. (*21*)] was amplified by PCR to incorporate *Nco*I and *Bam*HI restriction sites at the 5′ and 3′ ends. This fragment was subcloned into pET11d (Stratagene) using the *Nco*I and *Bam*HI restriction sites. The resulting microplasminogen will contain extra Met-Ala residues at the N-terminus. In addition, we substituted Cys536 and Cys541 with Ala and Ser, respectively. These two Cys residues are left without their native disulfide bond partners in microplasminogen (*22*), and we reasoned that they might interfere with the folding of microplasminogen by forming nonnative disulfide bonds. The microplasminogen sequence of the resulting construct was sequenced to ensure that no undesired mutations were inserted. The Arg561Ala mutation was created using the QuikChange method (Stratagene). The resulting plasmids, pET11dMplgR and pET11dMplgA, were transformed into *E. coli* strain BL21-(DE3). For protein isolation, a 10 mL overnight culture was diluted into 1 L of LB medium containing 100 μg/mL ampicillin. Protein expression was induced by the addition of 0.4 mM IPTG when the optical density of the culture reached 0.6−0.8. The cells were harvested by centrifugation after 6 h. The cell pellet was resuspended into 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 5% sucrose and disrupted by sonication at 4 °C. Microplasminogen was found in

inclusion bodies. Inclusion bodies were harvested by centrifugation at 7000 rpm (JA20 rotor) for 25 min at 4 °C. The inclusion bodies were washed twice by incubation with 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1% Triton X-100, and 2 M urea for 2 h. The inclusion bodies were solubilized in 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10 mM DTT, and 6 M GdnHCl. The protein was refolded by dilution into 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 5 mM L-cysteine, 0.05 mM L-cystine, and 3.5 M GdnHCl and dialyzed against two changes of 4 L of 100 mM Tris-HCl, pH 8.0, and 10 mM EDTA. Aggregates were removed by gel filtration chromatography on a BioGel P60 column (Bio-Rad) in 10 mM EDTA and 50 mM Tris-HCl, pH 8.0. The protein solution was concentrated by ultrafiltration to 4 mg/mL. Microplasminogen was stored at −20 °C in 50% glycerol. Microplasminogen was treated with equimolar SK; the resulting active sites were titrated with MUGB (*16*). This experiment demonstrated that >95% of the microplasminogen can be activated, which indicates that the microplasminogen is correctly folded.

Amidolytic Activity of Plasminogen by SK·Plgn. D-Val-Leu-Lys-pNA (S2251) is a good substrate of plasmin, SK·Plgn*, and SK·Pm; therefore, the activity of all three of these enzymes is determined by monitoring the hydrolysis of this substrate. Two experimental conditions were investigated: (a) 10 mM Hepes, pH 7.4, where the optimal activity of SK·Plgn* is observed (*7*) and (b) 100 mM NaCl and 50 mM Tris-HCl, pH 7.6; this buffer was chosen to approximate physiological salt concentrations. Similar results were obtained in both buffers, although lower rates were observed in the Tris/NaCl buffer as expected. The amidolytic activity of the SK complexes was monitored by adding SK (10−100 nM, depending on the temperature) to equimolar plasminogen and 500 μM D-Val-Leu-Lys-pNA. The reactions were monitored at 410 nm in a Hitachi U2000 spectrophotometer. Temperature was controlled by a circulating water bath. Less than 10% of the D-Val-Leu-Lys-pNA was consumed during the course of the reaction. The production of *p*-nitroaniline is linear under these conditions, and activity is determined by simple linear regression of the data.

Plasminogen Activator Properties of SK. Plasminogen activation was monitored by adding SK (2−20 nM, depending on the temperature) to 200−500 nM plasminogen and 500 μM D-Val-Leu-Lys-pNA in both buffer systems. This assay follows the production of plasmin by monitoring the change in the rate of hydrolysis of D-Val-Leu-Lys-pNA. The data were fit to equation in the form of $P = at + bt^2/2$, where P denotes *p*-nitroaniline, t is time, a is the initial velocity for D-Val-Leu-Lys-pNA hydrolysis by SK·Plgn*, and $b = A\{k_{plgn}[Plgn]/(K_{plgn}+[Plgn]\}$, where A is the specific activity of plasmin assayed by the hydrolysis of 500 μM D-Val-Leu-Lys-pNA, k_{plgn} is the catalytic rate constant for the conversion of Plgn to Pm by SK·Plgn, and K_{plgn} is the Michaelis constant for plasminogen activation [nomenclature of Wohl et al., (*23*)]. This equation is not corrected for the competition between substrate plasminogen and D-Val-Leu-Lys-pNA for SK·Plgn*. Since the concentration of D-Val-Leu-Lys-pNA is approximately the K_m value, the true value of K_{plgn} would be lower by approximately a factor of 2 ($1 + [S]/K_m = 2$) (*23*). Comparable results were obtained in both buffer systems.

Inhibition of SK·Plgn Formation by ΔIle1−SK*. Plasminogen (50 nM) was added to SK (50 nM) and varying concentrations of ΔIle1−SK at 4 °C in ΔIle1−SK in 500 μM D-Val-Leu-Lys-pNA. Under these conditions, all of the plasminogen will be bound to either SK or ΔIle1−SK. Since only the complex with SK has amidolytic activity, the fraction of plasminogen bound to SK can be determined by the rate of D-Val-Leu-Lys-pNA hydrolysis and the fraction of plasminogen bound to ΔIle1−SK can be determined by the fraction inhibition. Using mass balance, $[Plgn]_0 = [SK\cdot Plgn] + [\Delta Ile1{-}SK\cdot Plgn]$, $[SK]_0 = [SK]_{free} + [SK\cdot Plgn]$ and $[\Delta Ile1{-}SK]_0 = [\Delta Ile1{-}SK]_{free} + [\Delta Ile1{-}SK\cdot Plgn]$. Since $K_{wt} = [Plgn]_{free}[SK]_{free}/[SK\cdot Plgn]$ and $K_{mut} = [Plgn]_{free}[\Delta Ile1{-}SK]_{free}/[\Delta Ile1{-}SK\cdot Plgn]$, $K_{wt}/K_{mut} = [\Delta Ile1{-}SK\cdot Plgn][SK]_{free}/[SK\cdot Plgn][\Delta Ile1{-}SK]_{free}$. Alternatively, K_{wt}/K_{mut} can be determined using the tight binding inhibitor treatment of Henderson (*24*), where $[\Delta Ile1{-}SK]_0/(1 - v_i/v_0) = [Plgn]_0 + K_{mut}[([SK]_{free} + K_{wt})/K_{wt}]v_0/v_i$

with the assumption that $[SK]_{free} \gg K_{wt}$.. A competitive binding treatment can also be utilized to calculate K_{wt}/K_{mut} (*25*). The three methods yielded estimates of K_{wt}/K_{mut} between 0.2 and 0.6.

Activation of Plasminogen by SK·Pm. Wild-type and ΔIle1−SK (14 nM) were incubated with plasmin (14 nM) at 4 °C for 4 min. The complex was added to varying concentrations of plasminogen, 100 mM NaCl, 50 mM Tris-HCl, pH 7.6, and 500 μM D-Val-Leu-Lys-pNA at 4 °C. The reaction was monitored at 410 nm in a Hitachi U2000 spectrophotometer. The data were fit to equation in the form of $P = at + bt^2/2$ as above.

RESULTS AND DISCUSSION

Characterization of ΔIle1−SK. Wild-type and ΔIle1−SK were produced as fusion proteins with maltose binding protein (*16*). SK was separated from the fusion protein by treatment with Factor Xa. The N-termini of wild-type and ΔIle1−SK were confirmed by N-terminal sequencing. Two assays were used to assess the effect of this mutation on SK function: (a) the activation of excess plasminogen by a catalytic amount of SK and (b) the amidolytic activity of equimolar mixtures of plasminogen and SK. The plasminogen activation assay monitors the production of *p*-nitroaniline from the hydrolysis of D-Val-Leu-Lys-pNA by plasmin. The rate of *p*-nitroaniline formation is linearly dependent on the concentration of plasmin, SK·Plgn* and/or SK·Pm. Since the plasmin concentration is increasing linearly with time, the formation of *p*-nitroaniline is a parabolic function of time, and is described by an equation in the form of $P = at + bt^2/2$ (*26*). Assays were performed at 37 °C, as typically found in the literature, and at 4 °C, where the conversion of SK·Plgn to SK·Plgn* is believed to be rate limiting (*5*, *7*). Two buffer conditions were tested: 10 mM Hepes, pH 7.4, where SK·Plgn* has the highest activity, and 100 mM NaCl and 50 mM Tris-HCl, pH 7.6, which approximates physiological salt concentrations. Comparable results were obtained under both conditions, although, as expected, the reaction rates are lower in the Tris-HCl buffer. In addition, experiments were performed with plasminogen purified from plasma (which contained a mixture of Glu-plasminogen and Lys-plasminogen) and commercially available Glu-plasminogen (which migrated as a single band on SDS−PAGE under

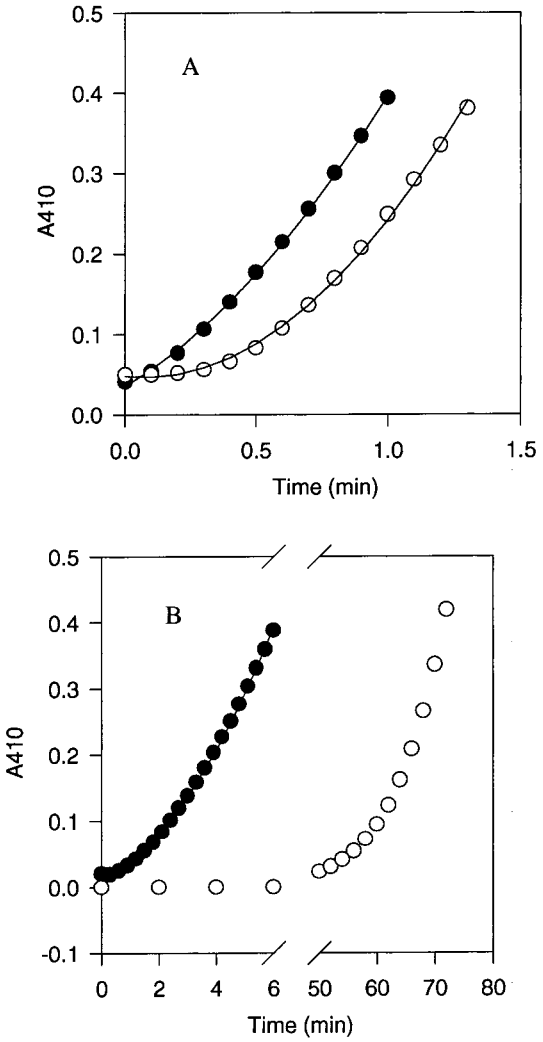

FIGURE 2: Activation of plasminogen by wild-type SK and ΔIle1−SK. The assays contained 500 μM D-Val-Leu-Lys-pNA in 10 mM Hepes, pH 7.4, and either wild-type SK (closed circles) or ΔIle1−SK (open circles). (A) Assays performed at 37 °C with 140 nM Glu-plasminogen and 2 nM SK. (B) Assays performed at 4 °C with 280 nM Glu-plasminogen and 20 nM SK. The solid lines are the fits to $P = at + bt^2/2$.

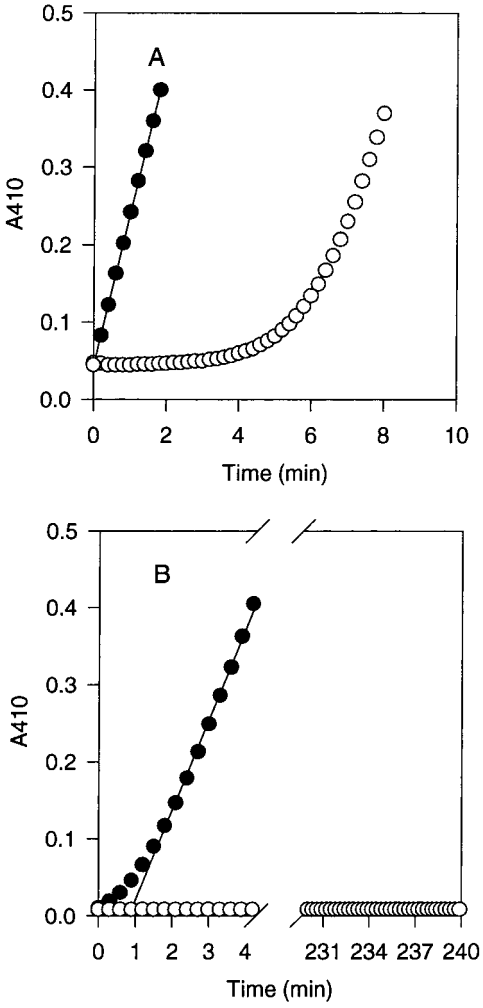

FIGURE 3: Amidolytic activity of the wild-type SK and ΔIle1−SK complexes with Glu-plasminogen. The assays contained 500 μM D-Val-Leu-Lys-pNA in 10 mM Hepes, pH 7.4, and either wild-type SK (closed circles) or ΔIle1−SK (open circles). (A) Assays performed at 37 °C with 14 nM Glu-plasminogen and 14 nM SK. (B) Assays performed at 4 °C with 100 nM Glu-plasminogen and 100 nM SK.

reducing conditions as shown below). Similar results were obtained with both plasminogen preparations. Only the experiments utilizing Glu-Plgn and Hepes buffer will be shown.

The Plasminogen Activator Activity of ΔIle1−SK Is Impaired. At 37 °C, the activation of plasminogen by catalytic amounts of wild-type SK displays a parabolic dependence on time as expected (*26*) (Figure 2A). ΔIle1−SK is also an efficient plasminogen activator at 37 °C (Figure 2A). Similar results have been reported with other truncated SK mutants (*16*−*18*). Thus, at 37 °C, the deletion of Ile1 does not appear to affect the activation of plasminogen by SK. However, careful inspection of the data suggests that the production of *p*-nitroaniline is no longer described by $P = at + bt^2/2$ in a meaningful way: while the fit of the data to this equation appears to be good, it yields a negative value for the a coefficient ($a = -0.03 \pm 0.1$) (Figure 2A). Systematic deviations are observed when a is constrained to 0. This observation suggests that the deletion of Ile1 may have changed the mechanism of plasminogen activation by SK.

In contrast, the deletion of Ile1 has a profound effect on the activation of plasminogen when the reaction is performed at 4 °C. Wild-type SK remains an efficient plasminogen activator at 4 °C, although the rate of activation is slower than at 37 °C (Figure 2B). As observed at 37 °C, there is a parabolic increase in *p*-nitroaniline concentration with time. However, plasminogen activation by ΔIle1−SK is severely decreased at low temperature; plasminogen activation is only observed after ∼1 h incubation (Figure 2B). Plasminogen activation by ΔIle1−SK is observed immediately upon warming of the cuvette (data not shown). These observations indicate that Ile1 is involved in the activation of plasminogen by SK. Since formation of SK·Plgn* is believed to be rate limiting at this temperature, the deletion of Ile1 may impair the formation of SK·Plgn*.

The Amidolytic Activity of the Plasminogen Complex with ΔIle1−SK Is Impaired. If Ile1 is required for the conversion of SK·Plgn to SK·Plgn* as proposed, then the deletion of Ile1 should impair the amidolytic activity of the SK-plasminogen complex. This prediction is confirmed by the experiments of Figures 3 and 4. Immediate hydrolysis of D-Val-Leu-Lys-pNA is observed when wild-type SK is mixed

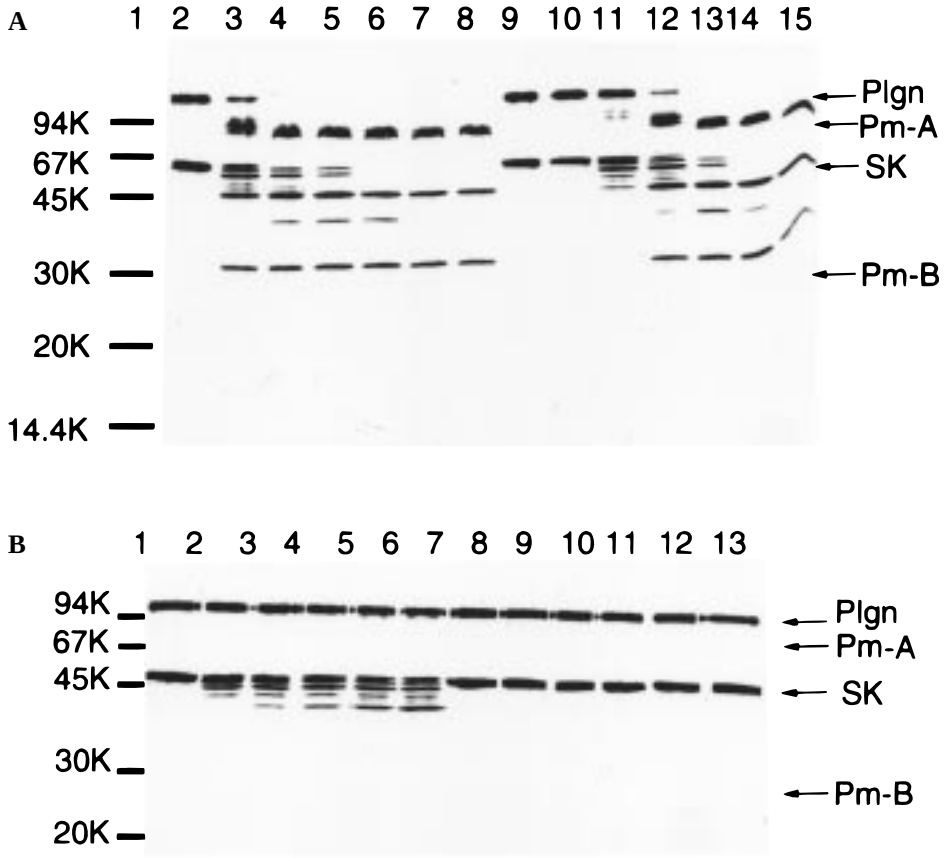

FIGURE 4: SDS−PAGE analysis of equimolar mixtures of SK and Glu-plasminogen. Glu-plasminogen (560 nM) was mixed with equimolar wild-type SK or ΔIle1−SK in 10 mM Hepes, pH 7.4. Aliquots were removed at various time intervals and the reaction was quenched with SDS sample buffer at 100 °C. Aliquots were analyzed by SDS−PAGE. (A) Reactions performed at 37 °C. Lane 1 contains the molecular weight standards. Lanes 2−8 contain aliquots removed from the reaction with wild-type SK at 0, 1, 2, 3, 5, 10, and 15 min, respectively. Lanes 9−15 contain aliquots removed from the reaction with ΔIle1−SK at 0, 1, 2, 3, 5, 10, 15 min, respectively. (B) Assays performed at 4 °C. Lane 1 contains the molecular weight standards. Lanes 2−7 contain aliquots removed from the reaction with wild-type SK at 0, 5, 10, 20, 35, and 60 min, respectively. Lanes 8−13 contain aliquots removed from the reaction with ΔIle1−SK at 0, 5, 10, 20, 35, and 60 min, respectively.

with plasminogen at 37 °C (Figure 3A). However, SDS−PAGE analysis reveals that plasminogen is converted to plasmin in less than 1 min under these conditions (Figure 4A). Thus, the amidolytic activity measured at 37 °C results from SK·Pm, not SK·Plgn*. When these experiments are performed at 4 °C, a lag of ∼1 min is observed in the production of *p*-nitroaniline (Figure 3B). SDS−PAGE analysis reveals that plasminogen is stable for over 20 min; conversion to plasmin is observed only after ∼45 min (Figure 4B). Thus, the amidolytic activity measured at 4 °C results from SK·Plgn*. This observation suggests that the lag is due to the slow formation of SK·Pgn*. These results confirm the observations of Chibber et al. (*7*).

When ΔIle1−SK is mixed with plasminogen at 37 °C, a lag of ∼5 min is observed in the production of *p*-nitroaniline (Figure 3A). SDS−PAGE analysis demonstrates that the conversion of plasminogen to plasmin parallels the development of amidolytic activity (Figure 4A). This observation suggests that the amidolytic activity results from ΔIle1−SK·Pm, not ΔIle1−SK·Plgn*. This hypothesis is supported when the experiment is performed at 4 °C (Figures 3B and 4B). No amidolytic activity is observed for at least 4 h, and no conversion of plasminogen to plasmin is observed.

*ΔIle1−SK Inhibits Formation of Wild-Type SK·Plgn**. Plasminogen activation and amidolytic activity will also be perturbed if the mutation prevents formation of SK·Plgn at 4 °C. Therefore, we designed an experiment to demonstrate that ΔIle1−SK can form a complex with plasminogen. If such a complex forms, it will prevent the formation of wild-type SK·Plgn*, thereby decreasing the amidolytic activity. Plasminogen was added to a solution containing equimolar wild-type SK and varying concentrations of ΔIle1−SK. As shown in Figure 5, amidolytic activity decreased with increasing ΔIle1−SK. This observation demonstrates that ΔIle1−SK·Plgn forms. Simple inspection of Figure 5 suggests that ΔIle1−SK has a slightly lower affinity for plasminogen than SK. If the affinity of SK and ΔIle1−SK were identical, 50% inhibition should be observed when the concentration of SK and ΔIle1−SK are equal; 20% inhibition is observed when both proteins are present at 50 nM. Values of K_d for SK·Plgn have been reported between 0.09 and 0.4 nM at ambient temperatures (*16*, *27*). Assuming a similar K_d at 4 °C, our experiments were performed under tight binding conditions, i.e., the concentrations of plasminogen and SK are greater than K_d. No free plasminogen will be present under these conditions, and the concentrations of unliganded SK and ΔIle1−SK can be determined by mass balance. The ratio of K_d for SK and ΔIle1−SK equals [SK]-[ΔIle1−SK·Plgn]/[ΔIle1−SK][SK·Plgn] = 0.2 ± 0.1. This experiment demonstrates that ΔIle1−SK binds to Plgn with slightly lower affinity than wild-type SK. This result is consistent with the observation that plasminogen can bind

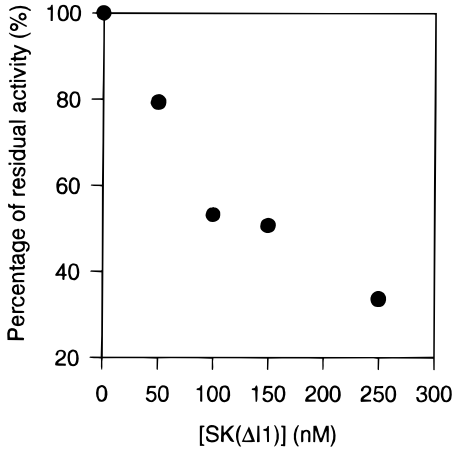

FIGURE 5: ΔIle1−SK inhibits formation of wild-type SK·Plgn*. SK (50 nM) was mixed with varying concentrations of ΔIle1−SK in 500 μM D-Val-Leu-Lys-pNA (S-2251) and 10 mM Hepes, pH 7.4, at 4 °C. The reaction was initiated with the addition of Glu-plasminogen (50 nM) and formation of wild-type SK·Plgn* was monitored by the presence of amidolytic activity (i.e., the change in absorbance at 410 nm).

with high affinity to SK fragments lacking the N-terminus (*15*, *16*, *18*). Therefore, the deletion of Ile1 must impair formation of SK·Plgn*.

Plasminogen Activation by SK·Pm Complexes. The molecular sexuality hypothesis predicts that the deletion of Ile1 will not perturb plasminogen activation by SK·Pm since pathway 2 bypasses formation of SK·Plgn* (Figure 1). As expected, the steady-state parameters for plasminogen activation by ΔIle1−SK·Pm are similar to those of wild-type SK·Pm at 4 °C: k_{cat} = 0.7 min^{-1} versus 0.5 min^{-1} and K_m = 0.9 μM versus 1.0 μM, respectively. In addition, the amidolytic activity of the ΔIle1−SK·Pm complex is indistinguishable from wild-type SK·Pm at 4 °C: K_m = 500 and 290 μM and k_{cat} = 10 and 6 s^{-1} for wild-type and ΔIle1−SK. Thus, Ile1 is not required for the activation of plasminogen by SK·Pm. This result also suggests that Ile1 is required for the conversion of SK·Plgn to SK·Plgn*.

The Amidolytic Activity of the Microplasminogen Complex with ΔIle1−SK Is Also Impaired. We expressed microplasminogen as described in Materials and Methods. This protein includes the protease domain of plasminogen, but is missing the kringle domains (*20*). Previous work has shown that microplasminogen can be activated by SK (*20*, *21*). The reactions of microplasminogen with wild-type SK are similar to those of plasminogen (Figures 6 and 7). Amidolytic activity is observed immediately upon the addition of wild-type SK to microplasminogen at 37 °C (Figure 6A). The Michaelis−Menten parameters for this reaction are k_{cat} = 33 s^{-1}, K_m = 300 μM, comparable to those reported in the literature (*21*). SDS−PAGE analysis reveals that microplasminogen is almost immediately converted to microplasmin under these conditions (Figure 7A), which suggests that this amidolytic activity results from SK·μPm.

As with plasminogen, a lag of 20 min is observed in the development of amidolytic activity when ΔIle1−SK is added to microplasminogen at 37 °C (Figure 6A). However, when the conversion of microplasminogen to microplasmin was monitored by SDS−PAGE, microplasmin was observed after only 1 min (Figure 7A). The SDS−PAGE experiment requires much greater concentrations of SK and microplasminogen than the amidolytic assay (560 nM versus 16 nM). Therefore, we reasoned that the increased concentrations of SK and microplasminogen are responsible for the more rapid conversion of microplasminogen to microplasmin in Figure 7A than Figure 6A. The magnitude of the lag in amidolytic activity does decrease as the concentrations of SK and microplasminogen increase; the lag is ~1 min when the concentrations of SK and microplasminogen are 560 nM (data not shown). This lag correlates with the conversion of microplasminogen to microplasmin in Figure 7A. Thus, the amidolytic activity appears to result from the formation of ΔIle1−SK·μPm.

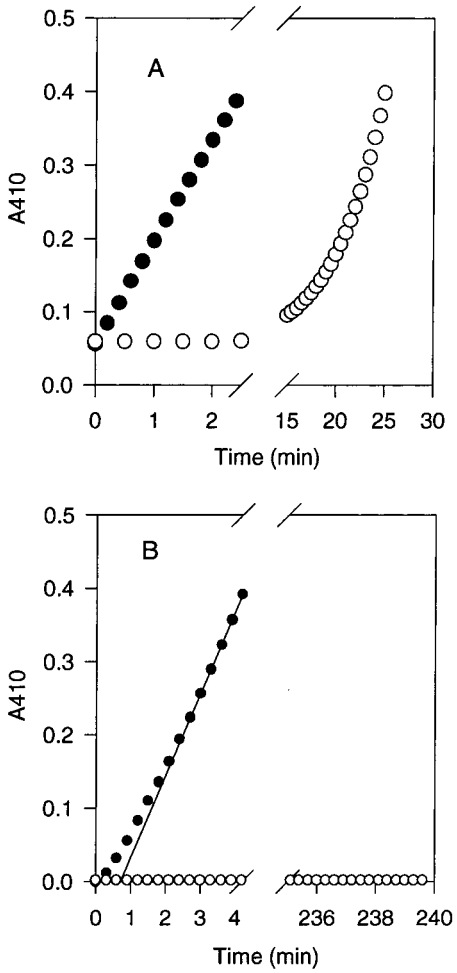

FIGURE 6: Amidolytic activity of the wild-type SK and ΔIle1−SK complexes with microplasminogen. The assays contained 500 μM D-Val-Leu-Lys-pNA in 10 mM Hepes, pH 7.4, and either wild-type SK (closed circles) or ΔIle1−SK (open circles). (A) Assays performed at 37 °C with 16 nM microplasminogen and 16 nM SK. (B) Assays performed at 4 °C with 100 nM microplasminogen and 100 nM SK.

The reactions of microplasminogen also mimic that of Glu-plasminogen at 4 °C. A lag of ~1.5 min is observed in the development of amidolytic activity when SK is added to microplasminogen (Figure 6B). This lag appears to be due to the slow formation of SK·μPgn* since the conversion of microplasminogen to microplasmin is observed only after 5 min (Figure 7B). No amidolytic activity is observed when ΔIle1−SK is added to microplasminogen at 4 °C (Figure 6B), and no conversion of microplasminogen to microplasmin is observed under these conditions (Figure 7B). These experiments demonstrate that microplasminogen is a good

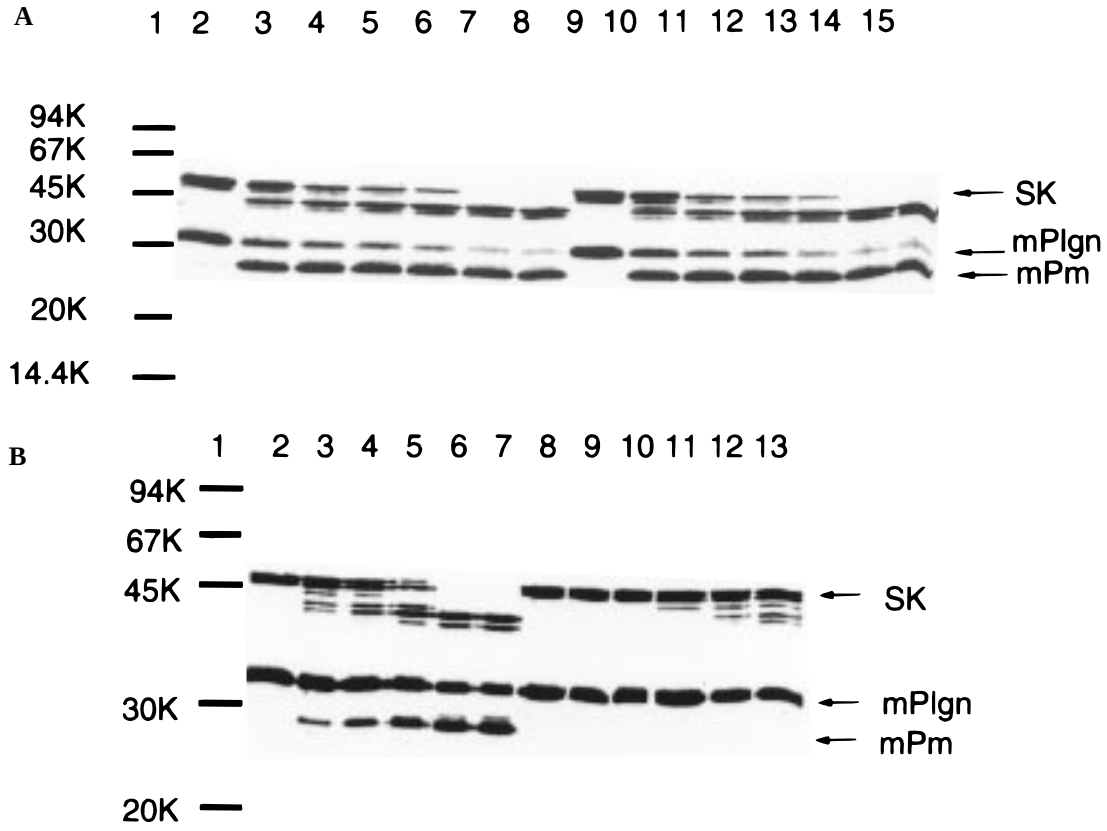

FIGURE 7: SDS−PAGE analysis of equimolar mixtures of SK and microplasminogen. Microplasminogen (560 nM) was mixed with equimolar wild-type SK or ΔIle1−SK in 10 mM Hepes, pH7.4. Aliquots were removed at various time intervals and the reaction was quenched with SDS sample buffer. Aliquots were analyzed by SDS−PAGE. (A) Reactions performed at 37 °C. Lane 1 contains the molecular weight standards. Lanes 2−8 contain aliquots removed from the reaction with wild-type SK at 0, 1, 2, 3, 5, 10, and 15 min, respectively. Lanes 8−15 contain aliquots removed from the reaction with ΔIle1−SK at 0, 1, 2, 3, 5, 10, and 15 min, respectively. (B) Reaction performed at 4 °C. Lane 1 contains the molecular weight standards. Lanes 2−7 contain aliquots removed from the reaction with wild-type SK at 0, 5, 10, 20, 45, and 60 min, respectively. Lanes 8−13 contain aliquots removed from the reaction with ΔIle1−SK at 0, 5, 10, 20, 45, and 60 min, respectively.

model for plasminogen and suggest that the amidolytic activity of ΔIle1−SK requires formation of ΔIle1−SK·μPm.

ΔIle1−SK Cannot Activate Arg561Ala−Microplasminogen. The above results suggest that ΔIle1−SK·Plgn must be converted to ΔIle1−SK·Pm in order to observe amidolytic activity. We tested this hypothesis by characterizing the reaction of SK and ΔIle1−SK with a mutant microplasminogen that cannot be converted to microplasmin. We expressed Arg561Ala−microplasminogen. The Arg561Ala mutation prevents cleavage of the 561−562 peptide bond so that microplasmin cannot form. Amidolytic activity is observed immediately upon the addition of wild-type SK to Arg561Ala−microplasminogen at 37 °C (Figure 8). The activity is similar to that observed in the experiments with wild-type microplasminogen (compare with Figure 6A). However, no amidolytic activity is observed when ΔIle1−SK is incubated with Arg561Ala−microplasminogen at 37 °C. The amidolytic activity of the ΔIle1−SK complex is at least 170-fold less than that of the wild-type complex. This observation indicates that the activity of ΔIle1−SK at 37 °C, and presumably the activity of other truncated SK mutants, requires the conversion of plasminogen to plasmin.

The Deletion of Ile1 Changes the Mechanism of Plasminogen Activation by SK. The experiments described above suggest that Ile1 is required for the conversion of SK·Plgn to SK·Plgn*. These observations are consistent with the molecular sexuality hypothesis whereby Ile1 of streptokinase

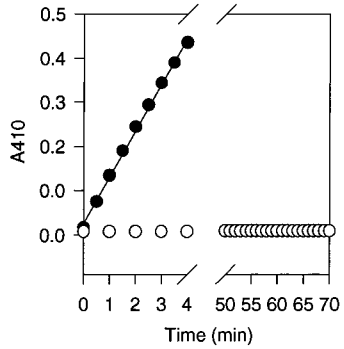

FIGURE 8: Amidolytic activity of the wild-type SK and ΔIle1−SK complexes with Arg561Ala−microplasminogen. The assays contained 500 μM D-Val-Leu-Lys-pNA in 10 mM Hepes, pH 7.4, and either wild-type SK (closed circles) or ΔIle1−SK (open circles). Assays performed at 37 °C with 16 nM microplasminogen and 16 nM SK.

substitutes for Val562 of plasmin. In the absence of Ile1, amidolytic activity, and presumably plasminogen activation, requires the formation of ΔIle1−SK·Pm. The mechanism of generation of ΔIle1−SK·Pm is unclear at this time. Plasmin often contaminates plasminogen preparations; it is possible that ΔIle1−SK·Pm initially forms from such adventitious plasmin. In this event, ΔIle1−SK-catalyzed plasminogen activation would follow pathway 2, in effect changing to a staphylokinase-like mechanism. From the

background reaction in the absence of SK, we estimate that no more than 0.1% of plasmin is present in the Glu-plasminogen preparation. This quantity of plasmin is not sufficient to account for the kinetics of plasminogen activation observed in Figure 2, which suggests that pathway 2 cannot account for the activity of ΔIle1−SK. However, it is quite possible that the SK·Pm proteolyzes SK·Plgn more rapidly than plasminogen alone. Indeed, the rapid conversion of SK·Plgn to SK·Pm at 37 °C is consistent with this proposal. Alternatively, while the activity of plasminogen is very low in comparison to plasmin, it nevertheless has some intrinsic activity. Therefore, ΔIle1−SK·Pm might form directly from the hydrolysis of ΔIle1−SK·Plgn by a second molecule of plasminogen or ΔIle1−SK·Plgn. In addition, SK is designed to bring two plasminogen molecules together. The close proximity of two plasminogen molecules in such a SK·Plgn·Plgn complex may accelerate the autoactivation of plasminogen, forming SK·Pm without the prior formation of SK·Plgn*.

The Kringle Domains Stabilize SK·Plgn but Are Not Required for the Rearrangement of SK·Plgn to SK·Plgn**. A ∼1 min lag is observed in the formation of SK·Plgn* at 4 °C (Figure 3B). Since SK·Plgn forms rapidly (see Figure 5), this lag must result from the rearrangement of SK·Plgn to SK·Plgn*. A similar lag is observed in the formation of SK·μPlgn* (Figure 6B). Since SK·Plgn* and SK·μPlgn* appear to be formed with similar rates, the kringle domains cannot be involved in the rearrangement of SK·Plgn to SK·Plgn*. Therefore, the slow step in the conversion of SK·Plgn to SK·Plgn* must be a change in the conformation of the protease domain.

In contrast, while SK·Plgn* is stable for at least 45 min at 4 °C, SK·μPlgn* is converted to SK·μPm in ∼5 min (Figures 4B and 7B). This observation suggests that the presence of the kringle domains stabilizes SK·Plgn*. It is possible that the kringle domains interact with the activation peptide and/or protease domain. Plasminogen exists in at least three different conformations, ranging from compact to open, depending on packing of the kringle and protease domains (*28*, *29*). This packing is controlled by the ligand state of the kringle domains. It seems reasonable to suggest that temperature will also control the conformational state of plasminogen, as well as the rate of interconversion of the conformations. It is likely that the temperature dependence of the conversion of SK·Plgn* to SK·Pm originates in the conformational stability of plasminogen.

*The Role of Lys698 in Formation of SK·Plgn**. Several researchers have proposed that Lys698 of plasminogen substitutes for Val562, forming a salt bridge with Asp740 in SK·Plgn* (*30*, *31*). This proposal originates in the structures of the single chain forms of human t-PA and vampire bat PA (*32*, *33*). Unlike most serine protease zymogens, the single chain forms of these proteases possess comparable activity to the mature enzymes. In both cases the analogous Lys156 forms a salt bridge with Asp194 (chymotrypsinogen numbering, Table 1). However, Lys156 is also found in several serine protease zymogens that are not intrinsically active. This observation indicates that Lys156 is not the sole determinant of zymogen activity, and structural studies suggest that residues 21 and 144 (chymotrypsinogen numbering) and the activation peptide also provide important interactions (*33*). Our results argue against formation of the Lys698−Asp740 salt bridge: the simplest explanation for the failure of ΔIle1−SK to form an active complex with Arg561Ala−μPlgn is that Ile1 of SK forms the salt bridge with Asp740. Alternatively, Ile1 of SK might be required for formation of a salt bridge between Lys698 and Asp740. Investigations are currently in progress to further define the role of Lys698 in the formation of SK·Plgn*.

Table 1: Chymotrypsinogen and Plasminogen Numbering

plasminogen numbering	chymotrypsinogen numbering
Arg561	Arg15
Val562	Val16
Lys698	Lys156
Asp740	Asp194
loop 692−695	150−153[a]

[a] Part of the autolysis loop, residues 142−153.

Implications of the SK·μPm Structure. The structure of SK and microplasmin has recently been reported, although the coordinates are not yet available (*30*). Unfortunately, this structure does not provide any information with respect to our proposal or to the alternate hypothesis that Lys698 substitutes for Val562. In microplasmin, Val562 forms the crucial salt bridge with Asp740, displacing the alternate counterion of SK·Plgn*, be it Ile1 of streptokinase, Lys698 of plasminogen, or some other residue. Lys698 is found near Asp740 in microplasmin, where it is adjacent to the 692−695 loop of plasminogen, which in turn forms an interface with streptokinase. The 692−695 loop is believed to be part of the activation domain of plasminogen by analogy to trypsinogen, and it seems likely that the interaction between this loop and streptokinase would help form SK·Plgn*. However, Lys698 would have to rotate in order to form the salt bridge with Asp740 in SK·Plgn*. Wang et al. (*30*) have hypothesized that the binding of streptokinase to this region of plasminogen triggers a change in position of Lys698 in SK·Plgn*. This mechanism would most likely require different conformations for streptokinase and the 692−695 loop of plasminogen as well as Lys698. It seems unlikely to us that streptokinase could bind effectively to two different conformations of plasminogen. In contrast, Ile1 could form the salt bridge with Asp740 to form SK·Plgn* while retaining the same structure as SK·μPm in the region of Lys698 and the 692−695 loop. We believe that this mechanism is more attractive than the Lys698 hypothesis because Val562 could displace Ile1 without disrupting the rest of the structure. Unfortunately, the first 12 residues of streptokinase are disordered in the SK·μPm structure, and without the coordinates it is impossible even speculate about the position of Ile1 and whether it can form the salt bridge. It also bears mentioning that until the structure of plasminogen is solved, the activation domain of plasmin is not defined. It is possible that the activation domain of plasminogen is different than that of trypsinogen.

Conclusions. The above experiments demonstrate that Ile1 of SK is required for the conversion of SK·Plgn to SK·Plgn*. In the absence of Ile1, the mechanism of plasminogen activation by SK requires the formation of SK·Pm. These results are consistent with the proposal that the N-terminus of SK forms a salt bridge with Asp740 of plasminogen, and thus provide evidence in favor of the molecular sexuality hypothesis.

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