Streptokinase Triggers Conformational Activation of Plasminogen through Specific Interactions of the Amino-terminal Sequence and Stabilizes the Active Zymogen Conformation

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The fibrinolytic proteinase plasmin (Pm)1 is formed by proteolytic cleavage of plasminogen (Pg) at Arg561-Val562 in plasminogen (Pg) generates plasmin (Pm) through a classical activation mechanism triggered by an insertion of the new amino terminus into a binding pocket in the Pg catalytic domain. Streptokinase (SK) circumvents this process and activates Pg through a unique nonproteolytic mechanism postulated to be initiated by the intrusion of Ile1 of SK in place of Val562. This hypothesis was evaluated in equilibrium binding and kinetic studies of Pg activation with an SK mutant lacking Ile1 (SK2–414). SK2–414 retained the affinity of native SK for fluorescein-labeled [Lys]Pg and [Lys]Pm but induced no detectable conformational activation of Pg. The activity of SK2–414 was partially restored by the peptides SK1–2, SK1–5, SK1–10, and SK1–15, whereas Pg562–569 peptides were much less effective. Active site-specific fluorescence labeling demonstrated directly that the active catalytic site was formed on the Pg zymogen by the combination of SK1–10 and SK2–414, whereas sequence-scrambled SK1–10 was inactive.

Cleavage of Arg561-Val562 in plasminogen (Pg) generates plasmin (Pm) through a classical activation mechanism triggered by an insertion of the new amino terminus into a binding pocket in the Pg catalytic domain. Streptokinase (SK) circumvents this process and activates Pg through a unique nonproteolytic mechanism postulated to be initiated by the intrusion of Ile1 of SK in place of Val562. This hypothesis was evaluated in equilibrium binding and kinetic studies of Pg activation with an SK mutant lacking Ile1 (SK2–414). SK2–414 retained the affinity of native SK for fluorescein-labeled [Lys]Pg and [Lys]Pm but induced no detectable conformational activation of Pg. The activity of SK2–414 was partially restored by the peptides SK1–2, SK1–5, SK1–10, and SK1–15, whereas Pg562–569 peptides were much less effective. Active site-specific fluorescence labeling demonstrated directly that the active catalytic site was formed on the Pg zymogen by the combination of SK1–10 and SK2–414, whereas sequence-scrambled SK1–10 was inactive. The characterization of SK1–10 containing single Ala substitutions demonstrated the sequence specificity of the interaction. SK1–10 did not restore activity to the further truncated mutant SK55–414, which was correlated with the loss of binding affinity of SK55–414 for labeled [Lys]Pm but not for [Lys]Pg. The studies support a mechanism for conformational activation in which the insertion of Ile1 of SK into the Pg amino-terminal binding cleft occurs through sequence-specific interactions of the first 10 SK residues. This event and the preferentially higher affinity of SK5–414 for the activated proteinase domain of Pm are thought to function cooperatively to trigger the conformational change and stabilize the active zymogen conformation.

The fibrinolytic proteinase plasmin (Pm)1 is formed by pro-
The unresolved roles of SK binding to Pg and Pm and interactions of the SK amino-terminal sequence with the Pg amino-terminal binding site in the conformational activation mechanism were evaluated in the present fluorescence and kinetic studies of Pg interactions with an SK mutant lacking Ile
sup>1
(SK
sup>2–414), SK
sup>2–414 bound to fluorescein-labeled [Lys]Pg and [Lys]Pm with affinities equivalent to native SK, but this tight binding alone was insufficient to induce conformational activation of Pg. Compelling evidence for an additional critical requirement for interactions of the SK amino-terminal residues was obtained in the novel demonstration that the loss of activity of SK
sup>2–414 could be restored partially by the specific binding of peptides based on the amino-terminal 10-residue sequence of SK. The results support a mechanism of conformational activation in which the amino-terminal sequence of SK beginning with Ile
sup>1 interacts in a sequence-specific manner with the amino-terminal binding cleft of Pg and through extended binding sites on the SK
sup>2–414-Pg complex to induce a transition toward the active conformation. Evidence for the additional role of preferential binding affinity of SK for Pm compared with Pg was obtained in the finding that conformational activity of the further SK truncation mutants SK
sup>55–414 and SK
sup>78–414 could not be restored by SK
sup>1–10. The loss of activity was associated with a selective loss of affinity of SK
sup>55–414 for labeled [Lys]Pg, whereas the affinity for [Lys]Pm was not significantly affected. On this basis, conformational activation is concluded to occur through the amino-terminal interaction in cooperation with the stabilization of the active conformation by the enhanced binding affinity of the remainder of SK for the active form of thezymogen.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Characterization**—[Glu]Pg and [Lys]Pg carboxylic acid forms 1 and 2 were purified and characterized by the procedures published previously (18–20). Pg preparations were 71–84% active as determined by active site titration (18). [Lys]Pg was generated from Pg cleavage of [Glu]Pg (19), and the reaction mixture was chromatographed on soybean trypsin inhibitor-agarose in 10 mM Hepes, 10 mM Mes, 20 mM 6-AHA, 0.15 M NaCl, 1 mg/ml of PEG, pH 7.4, to remove Pg. [Lys]Pg was further purified by chromatography on amine- or diol-Sepharose (21). [Lys]Pg and [Lys]Pm form 2 were labeled at the catalytic site with ATA-FFR-CH
sub>2Cl and 5-(iodoacetamido)fluorescein to give [5F]FFR-[Lys]Pg and [5F]FFR-[Lys]Pm following the methods described previously (18, 20). The polymerase chain reaction was used to generate SK lacking the codon for Ile
sup>1 (SK
sup>2–414). The 5′ and 3′ primers were TCATCTATCATAT-GGTCGACCCTAGTGGTGCTG and AAGGTTACCGAACATC, respectively. Template DNA was pET-3a/SK (24). The resulting product was digested with NdeI and AflII and then ligated into similarly restricted pET-3a/SKC. An analysis of a single clone (pET-3a/SK
sup>2–414) confirmed the absence of unintended mutations in the SK sequence bounded by the NdeI and AflII sites. SK
sup>2–414 was expressed in BL21(DE3)lysS and purified as described previously (24). Protein sequence analysis confirmed that the amino terminus of the recombinant protein was alanine.

Protein concentrations were determined by absorbance at 280 nm using the following absorption coefficients (mg/ml)
sub>–1 cm
sup>–1): SK and SK
sup>2–414, 0.95 and 47,000 (25, 26); SK
sup>55–414, 0.93 and 40,800; SK
sup>78–414, 0.80 and 38,400; [Lys]Pg, 1.69 and 84,000; and [Lys]Pm, 1.9 and 84,000 (18–20). The absorption coefficient for SK
sup>2–414 was calculated from the aromatic amino acid content (27).

**Peptides**—The peptides were SK
sup>1–15 (IAGPEWLLDRFVPNV), SK
sup>1–10, SK
sup>1–5, SK
sup>2–14, a sequence-scrambled SK
sup>1–10 control peptide (ELGLDPWIAAR), the dipeptides Ile-VaI, Ac-Ile-VaI, Phe-VaI, Val-VaI (Pg
sup>92–95a), the peptides Pg
sup>92–96 and Pg
sup>92–95a with Cys
sup>96 replaced by Ser (Pg
sup>92–96, C568S), VGFGSSVAT, and SK
sup>1–10 peptides with single Ala substitutions. All peptides except Ile-VaI, Phe-VaI, and Ile-Ala (SK
sup>2–14) were synthesized by SynPep (Dublin, CA) and were >95% pure.

**Fluorescence Studies**—Fluorescence titrations were done with an SLM 8100 spectrofluorometer following methods described previously (18, 19) with 500 nm excitation and 516 nm emission (8 or 16 nm bandpass) in 50 mM Hepes, 0.125 mM NaCl, 1 mM EDTA, 1 mg/ml of PEG, 1 mg/ml of bovine serum albumin, 1 μM FFR-CH
sub>2Cl, pH 7.4, and at 25 °C. Blank-corrected titrations of [5F]FFR-[Lys]Pg and [5F]FFR-[Lys]Pm, expressed as the fractional change in the initial fluorescence (F
sub>o) vs. [5D](F/F
sub>o)) versus the total SK concentration, were analyzed by nonlinear least-squares fitting of the quadratic binding equation (23) with Scientist software (MicroMath). The fitted parameters were the maximum fluorescence change and dissociation constant with the stoichiometric factor fixed at one or the previously determined value for that interaction. Uncertainties in reported parameters are ±2 S.D.

**Plasminogen Activation Kinetics**—The activation of [Lys]Pg form 1 by SK species was measured by continuously monitoring the hydrolysis of 200 μM D-t-Val-Leu-Lys-p-nitroanilide (<5% substrate depletion) at 405 nm and 25 °C in 50 mM Hepes, 0.125 mM NaCl, 1 mM EDTA, 1 mg/ml of PEG, ± 10 or 50 mM 6-AHA as indicated, pH 7.4. For native and wild-type SK, the reactions were typically initiated by the addition of SK2–414 (5 mM [Lys]Pg) to 5 mM [Pf]Pg. For mutant SK species, additional reactions were started by the addition of 500 mM SK mutant to 100 mM [Lys]Pg. For reactions in the presence of peptides, [Lys]Pg was preincubated for 5 min before initiation with the SK mutant. The slow background rate obtained with Pg alone was subtracted, and the progress curves, measured by the absorbance change (ΔA
sub>280nm), were fit by the parabolic Equation 1 (28, 29) as follows.

\[
\Delta A_{280nm} = \frac{V_f}{2} + V_f \quad (\text{Eq. 1})
\]

This analysis gave the initial rate of chromogenic substrate hydrolysis at the beginning of the reaction (V
sub>i) because of the conformationally activated SKP
sup>g (where P
sup>g represents conformationally activated Pg) complex and the rate of the increase in activity with time due to Pm formation (V
sub>f). For reactions in the presence of 6-AHA that were linear, V
sub>i was obtained from the slopes of the least squares fits of the absorbance versus time data. The potencies of the peptides were determined from the least squares fitted slopes of linear plots of V
sub>i as a function of peptide concentration. The effect of 2 mM SK
sup>1–10 on chromogenic substrate hydrolysis by the SK-Pm complex was measured from rates of reaction of 7 mM Pm after preincubation for 2–3 min with 58 mM wild-type SK, native SK, SK
sup>2–414, or 200 mM SK1–10 or SK
sup>2–414 with 500 nM SK2–414. These SK complexes were then reactivated with 5 mM [Pf]Pg and the rate of the increase in activity with time due to Pm (ΔA
sub>280nm) was measured. Uncertainties in reported parameters are ±2 S.D.

**RESULTS AND DISCUSSION**

**Binding of SK
sup>2–414 to Pg and Pm**—The binding of SK
sup>2–414 to fluorescent analogs of Pg and Pm that were specifically labeled at the catalytic site with ATA-FFR-CH
sub>2Cl and 5-(iodoacetamido)fluorescein (18, 19) ([5F]FFR-[Lys]Pg and [5F]FFR-[Lys]Pm) was evaluated to determine whether the affinities for Pg and Pm were affected by the mutation. Titrations of fluorescein-labeled [Lys]Pg and [Lys]Pm with SK
sup>2–414 yielded dissociation constants of 31 ± 7 mM and 20 ± 12 μM, which were indistinguishable from those determined previously for native SK and...
[Lys]Pg of 44 ± 9 nM and the much tighter value of 11 ± 2 pm for [Lys]Pm (18, 19) (Fig. 1). The binding of recombinant wild-type SK was similarly indistinguishable from native SK with a dissociation constant for [Lys]Pg of 77 ± 12 nM (data not shown) and the previously determined value for [Lys]Pm of 33 ± 6 pm (18). The maximum fluorescence change observed for labeled Pm upon binding SK2–414 was −18 ± 2%, which was considerably less than the −52 ± 1% observed for native SK and −49 ± 1% for wild-type SK (18), indicating significant differences in the active site environments of the SK–Pm and SK2–414–Pm complexes. In contrast to the results for Pm, SK2–414 binding to labeled Pg resulted in a maximum change in fluorescence of −24 ± 1%, which was indistinguishable from that of native SK (−28 ± 1%). These results showed that the lack of activity previously observed for SK2–414 (13, 14) was not attributed to the loss of affinity for [Lys]Pg or [Lys]Pm, and that the amino-terminal Ile residue did not contribute significantly to binding affinity. These results were in approximate agreement with the finding of a 5-fold reduced affinity for SK2–414 compared with native SK, estimated from the kinetics of [Glu]Pg activation (13). However, Ile1 was required to induce the full perturbation in the active site of Pm that accompanied the binding of SK as judged by the difference in the maximum fluorescence change.

Conformational Activation of Pg by SK2–414 and SK1–10—The activity of SK2–414 in plasminogen activation via the conformational change in Pg and the effect of synthetic peptides based on the SK amino-terminal sequence were examined in the kinetic studies of [Lys]Pg activation. Reactions were measured by continuous monitoring of the hydrolysis of the plasmin substrate H-o-Val-Leu-Lys-p-nitroanilide. As previously demonstrated (28, 29), the addition of native SK to 5–20 nM Pg resulted in a parabolic increase in product concentration with time, which could be analyzed by the fitting of Equation 1 (see under “Experimental Procedures”) to extract the initial rate of chromogenic substrate hydrolysis (V1) because of rapid conformational activation of Pg and the subsequent slower rate of proteolytic conversion of Pg to Pm (V2) from the acceleration phase. The dependence of V1 on chromogenic substrate concentration at saturating levels of SK showed Michaelis-Menten kinetics with parameters for the activated SK–Pg* complex of 37 ± 11 s−1 for kcat and 3.0 ± 1.1 mM for km (data not shown).

As observed previously (13), the reactions of Pg with saturating levels of SK2–414 yielded progress curves with no initial activity followed after several minutes by rapid acceleration where the full-time course was not well described by Equation 1. Although this apparent change in mechanism is not completely understood, the acceleration phase has been previously shown to involve Pm formation (13) and may involve traces of Pm in Pg preparations that binds to SK2–414 and proteolytically activates Pg. This activation pathway has been suggested (13) to account for previous reports that SK species modified at the amino-terminus retained activity (15, 16). Investigation of this secondary process was not pursued further, and the studies were focused on V1, which was the rate of interest in conformational activation of Pg.

The activity of SK2–414 was tested further at high sensitivity in kinetic studies at 100 nM [Lys]Pg and 500 nM SK2–414 in the absence and presence of the SK peptide SK1–10 (Fig. 2). Limiting the assays under these conditions to the initial phase of Pg activation resulted in progress curves that were well described by Equation 1 and allowed V1 to be determined accurately (Fig. 2). SK2–414 alone had no detectable activity in conformational activation under these conditions as shown by the intercept of the linearly transformed data, corresponding to a value for V1 that was indistinguishable from zero (Fig. 2; inset). An addition of 1 mM SK1–10, however, increased V1 dramatically indicating that the activity of SK2–414 in conformational activation was partially restored by the peptide (Fig. 2).

Effect of Aminoterminal Peptides on Conformational Activation of Pg by SK2–414—The specificity of the binding site for amino-terminal peptides was first characterized in similar kinetic studies of the effect of various peptides on conformational activation of [Lys]Pg by SK2–414. SK1–2, SK1–5, SK1–10, and SK1–15 increased V1 linearly with increasing peptide concentration (Fig. 3A), indicating that the regained function in conformational activation was associated with a weak binding of the peptides to the SK2–414–Pg complex. The effectiveness of the peptides increased with increasing length past the amino-terminal dipeptide sequence through at least SK15 (Fig. 3A). On the basis of the kinetic parameters determined for the SK–Pg complex, SK1–10 and SK1–15 at 1 mM supported a regain of 3.4 ± 1% and 11 ± 1% of the activity of native SK, respectively. Peptides corresponding to the Pg sequence, which normally inserts into the amino-terminal binding pocket upon proteolytic conversion to Pm, Pg562–563, Pg562–565, and...
Plasminogen Activation

Fig. 3. Dependence of conformational activation of [Lys]Pg by SK2–414 on amino-terminal peptides of SK and Pg. A, the initial rate of chromogenic substrate hydrolysis (Vt) expressed as ΔA$_{405}$/t is plotted for reactions of 100 mM [Lys]Pg and 500 mM SK2–414 as a function of total peptide concentration ([Peptide]) for SK1–10 (○), SK1–10 (●), SK2–414 (asterisk), Pm$_{562–569}$ (C566S) (■), Pm$_{562–565}$ (△), Pm$_{562–563}$ (◊), Ile-Val (+), Ac-Ile-Val (□), and Phe-Val (○). Solid lines represent linear fits to the data. B, Vt is plotted for SK2–414 reactions in the absence (△) and presence (□) of 50 mM 6-AHA and for SK55–414 (○) and SK562–565 (●) in the absence of 6-AHA as a function of SK1–10. Also shown are the results for SK2–414 as a function of sequence-scrambled SK1–10 concentration in the absence (asterisk) and presence (□) of 6-AHA. Assays were performed and analyzed as described under “Experimental Procedures.”

Fig. 4. Active site-specific fluorescence labeling of the catalytic site of [Lys]Pg in the presence of SK2–414 and SK1–10. Fluorescence-visualized (A and C) and Coomassie Blue-stained (B and D) SDS gels run under nonreducing (lanes 1–4) and reducing conditions (lanes 5–8) of samples (4 μg) from fluorescence-labeling reactions of [Lys]Pg with SK2–414 in the absence (A and B) and presence (C and D) of 6-AHA as described under “Experimental Procedures.” Bands corresponding to reduced Pg (PgR), nonreduced Pg (PgN), SK2–414 (SK), and the light chain of Pm (Pm-I) are indicated. The migration positions of protein standards are shown along with the molecular weights in thousands. Samples were [Lys]Pg alone (lanes 1 and 8), reaction products of [Lys]Pg with SK2–414 in the presence of 3 mM SK1–10 (lanes 2 and 5), reaction products in the presence of 3 mM sequence-scrambled SK1–10 (lanes 3 and 6), and reaction products in the absence of peptides (lanes 4 and 7).

Pm$_{562–569}$ (C566S), showed less activity than the SK peptides and no significant increase with increasing length (Fig. 3A). Pm$_{562–568}$ (C566S), for example, was <5% as effective as SK1–10 in restoring conformational activation. A sequence-scrambled peptide of identical composition to SK1–10 induced no increase in Vt, indicating the sequence specificity of the interaction of SK2–414 with SK1–10 (Fig. 3B). In other control experiments, SK1–10 had no significant effect on the kinetics of Pg activation by native SK or on the chromogenic substrate activity of the SK-Pm complex (data not shown).

Direct Demonstration of Activation of the Pg Catalytic Site by SK2–414 and SK1–10—Active site-specific fluorescence labeling was used to confirm the identity of the active species formed in reactions of Pg with SK2–414 and SK1–10. Reaction mixtures containing [Lys]Pg and SK2–414 in the absence or presence of 3 mM SK1–10 or the presence of the same concentration of scrambled SK1–10 were inactivated with ATA-F FR-CH$_2$Cl. The thiol group subsequently generated on the incorporated inhibitor was specifically labeled with 5-(iodoacetamido)fluorescein, and the reaction products were examined by SDS gel electrophoresis (Fig. 4). Covalent active site-specific labeling of Pg was observed in the presence of SK1–10 as evidenced by the intensely fluorescent band corresponding to Pg in the reduced sample (Fig. 4, A and B, lanes 5), whereas a less intense band was also present that corresponded to the labeled light chain of Pm formed during the experiment (Fig. 4 A and B, lanes 6 and 7). Although the control reactions showed a significantly lower fluorescence incorporation, essentially all of the Pg in these reactions were converted to Pm at the high protein concentrations necessary for the experiment (Fig. 4B). Therefore, similar reactions were performed in the presence of 50 mM 6-AHA to inhibit proteolytic activation (30). It was first confirmed that under these conditions SK1–10 activated SK2–414 with indistinguishable potency as that seen in the absence of 6-AHA and also that the scrambled peptide was inactive (Fig. 3B). Pg was not significantly converted to Pm under these conditions, and the Pgzymogen was intensely labeled in the presence of SK1–10 as before, whereas control reactions with scrambled SK1–10 or no peptide resulted in no significant labeling (Fig. 4, C and D). These results clearly identified the intact Pgzymogen as the initially formed active species in the absence and presence of saturating 6-AHA and demonstrated that conformational activation required both SK2–414 and SK1–10.

Sequence Specificity of the SK2–414 Interaction—Previous studies (4) of trypsinogen demonstrated the specificity of the amino-terminal binding cleft for dipeptides in which the natural Ile-Val sequence bound most tightly and the extension of the peptide to three residues had no further effect. As shown by the results in Fig. 3A, the specificity of the analogous interaction of [Lys]Pg with dipeptides was characterized by very low but reproducible enhancements in the activity of comparable magnitude for Ile-Val and Ile-Ala (SK1–10). Ac-Ile-Val and Phe-Ile had no detectable activity, as predicted from the studies of trypsinogen, because of the lack of a free amino terminus and steric hindrance, respectively (4). Interestingly, the natural sequence in [Lys]Pm (Val-Val) was also inactive compared with Ile-Val, similar to the large difference in affinity observed for trypsinogen (4). These results supported the involvement of the amino-terminal binding site of Pg in the activation of the SK2–414-Pg complex by SK1–10 and suggested that the site had a trypsinogen-like specificity for dipeptides.

To address the specificity of the interaction of SK1–10 in more detail, wild-type and nine single Ala-substituted SK1–10 peptides were compared in conformational activation in the ab-
The activities of SK2–10 peptides containing Ala substitutions at the residues indicated were determined relative to the wild-type peptide with Ala at residue 2. The slopes of the activity of SK2–414 as a function of peptide concentration are shown for reactivation in the absence (solid bars) and presence (open bars) of 10 or 50 mM 6-AHA. The error bars represent the 95% confidence intervals. Experiments were performed, and the data were analyzed as described under “Experimental Procedures.”

By contrast, SK55–414 bound to [Lys]Pg with a dissociation potency-enhancing effect of 2-fold, suggesting a negative role for SK1–10 peptides. SK55–414 for [5F]FFR-[Lys]Pg was determined from fluorescence titration at 15 nM [5F]FFR-[Lys]Pg (●). The solid line represents the least squares fit with the dissociation constant given in the text. The dashed line shows a simulation of native SK binding to [5F]FFR-[Lys]Pg for comparison, calculated using the parameters previously determined (18, 19). Fluorescence titrations were performed and analyzed as described under “Experimental Procedures.”

The results of these studies support the molecular sexuality hypothesis (5, 13) and extend the understanding of the conformational activation mechanism in the conclusion that the combination of sequence-specific interactions of SK1–10 with the amino-terminal binding cleft of Pg and enhanced binding affinity of SK2–414 for the activated conformation of the Pg catalytic domain are both critical elements of the mechanism. As expected from previous studies of trypsinogen and Ile-Val (4, 5), the combination of SK1–10 and SK2–414 was less effective in Pg activation than native SK. This was consistent with the apparently low intrinsic affinity of the amino-terminal interaction and the loss of proximity and orientation available to the SK sequence in the intact molecule. Unlike previous studies of this site in trypsinogen, however, the SK peptides increased in potency with increasing length beyond the dipeptide and were significantly more effective than comparable Pg peptides. Moreover, an analysis of SK2–414 reactivation by each of wild-type and nine Ala-substituted SK1–10 peptides demonstrated that not only Ile1 but also Gly3 and, to a lesser extent, Pro2, Trp6, and Arg10 played significant roles in determining the specificity of the peptide interaction. On this basis, the results are concluded to reflect the specificities of the interactions of the SK1–10 sequence with sites that extend beyond the amino-terminal dipeptide binding site. It is important to consider that not only Ile1 but also Gly3 and, to a lesser extent, Pro2, Trp6, and Arg10 play significant roles in determining the specificity of the peptide interaction. On this basis, the results are concluded to reflect the specificities of the interactions of the SK1–10 sequence with sites that extend beyond the amino-terminal dipeptide binding site. It is important to consider the possible effect of the 2–10 residue sequence present on SK2–414 in activation by the peptides. The removal of Ile1 in SK5–414 that normally anchors the amino-terminal binding interaction may result in weakening the binding of the remaining SK2–10 sequence to Pg or disordering of the SK2–10 segment. The latter possibility is suggested by the disordered structure of this sequence when SK is bound to micro-Pm where Val562 occupies the amino-terminal binding site and Ile1 does not interact (17). In either case, the results would reflect the specificities of the amino-terminal binding site on Pg in the SK2–414–Pg complex, although the possibility that the peptides interact with the SK component of the complex cannot be ruled out completely. The SK1–10 sequence specificity of the amino-terminal interaction is suggested to contribute to the narrow specificity of SK for the activation of Pg among homologous zymogens.

The fluorescence probe in [5F]FFR-[Lys]Pm is thought to be in or near the S4 substrate specificity subsite. Previous studies with similar tripeptide fluorescence probes and chromogenic substrates demonstrated that the probes did not report changes affecting S1 but reported effects on the specificity of the S2 subsite, and these changes contributed to those reported by the probes located in the vicinity of S4 (18, 19). Thus, the...
sources of the fluorescence changes include changes in the environment of the S2-S4 subsites, if not other events as well. In this context, the dissimilar maximum fluorescence changes observed with SK2–414 and native SK binding to [5F]FFR-[Lys]Pm but not for [5F]FFR-[Lys]Pg are thought to represent Ile1-dependent differences in the subsite perturbations of Pg and Pm. Further studies will be needed to resolve the net fluorescence changes accompanying SK binding into the individual sources of the perturbations.

The inability of SK1–10 to complement the activity of SK55–414 and SK78–414 compared with SK2–414 was correlated with a 360-fold reduced affinity of SK55–414 for labeled [Lys]Pm (18), whereas the affinity for labeled [Lys]Pg was not significantly affected. This indicated that the interactions of residues 3–54 of the SKa-domain were not essential for Pg binding but were essential for both preferentially tighter binding of SK to Pm and for conformational activation of Pg. Together, the results support a mechanism in which the insertion of Ile1 of SK into the amino-terminal binding cleft acts as the trigger to initiate the transition of Pg toward the active conformation. This is thought to occur in cooperation with preferential stabilization of the active zymogen species as a result of the higher affinity of SK for the activated conformation of the Pg catalytic domain.

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