Streptokinase Binds to Human Plasmin with High Affinity, Perturbs the Plasmin Active Site, and Induces Expression of a Substrate Recognition Exosite for Plasminogen

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**Streptokinase (SK),** a 47,000 molecular weight protein from Streptococcus equisimilis, is used as a thrombolytic drug to activate plasminogen (Pg) into plasmin (Pm), the serine proteinase responsible for dissolution of fibrin clots. SK possesses no intrinsic enzyme activity, but binds specifically to Pg and Pm, converting both the zymogen and proteinase into Pm activators. SK binding to Pm results in the conformational expression of an active catalytic site on the zymogen that cleaves specifically the Arg561-Val562 activation bond in the catalytic domain of Pm to form Pm (3–7). SK also binds to Pm, transforming the substrate specificity of the proteinase from one which is incapable of Pm activation into an active catalyst (7–9), and decreasing greatly the reactivity of Pm toward its physiological serpin inhibitor, α2-antiplasmin (10). The mechanism of conformational activation of Pg and the origin of the dramatic macromolecular substrate specificity change that Pm exhibits upon SK binding are unknown. The x-ray crystal structure of SK bound to the catalytic domain of Pm shows that SK consists of three, similarly-folded globular domains (11). SK surrounds the catalytic site, the three domains forming a “three-sided crater” with the active site of Pm at the bottom (11). The structure suggests that SK may induce a conformational change affecting the specificity of the catalytic site and/or participate directly in the binding of Pm as a substrate (11). However, the contribution of these two mechanisms to the change in Pm specificity has not been established. Structure-function correlation studies with recombinant or proteolytic SK derivatives support a direct role for SK in recognition of Pg as a substrate (12–14). SK has been reported to cause modest changes in the kinetic constants for Pm with two peptide chromogenic substrates, suggesting that SK binding may also cause a change in catalytic specificity (9, 15, 16).

Quantitative equilibrium binding studies of SK interactions with Pg and Pm are required to define the mechanism of Pm activation and the origin of the SK-induced change in Pm substrate specificity. These studies are complicated by the coupling between the binding interactions and formation of Pm, and the ensuing additional proteolytic reactions catalyzed by Pm. Pm cleaves a 77-residue peptide from the amino terminus of native [Glu]Pg to form [Lys]Pg, which is accompanied by a conformational transition from a compact to an extended conformation that is activated to Pm more rapidly (2, 17, 18), and binds SK with enhanced affinity (19). SK also undergoes proteolysis by Pm, with early cleavage at Lys59 generating modified SK (SK1), which consists of the noncovalent complex between the SK1–59 and SK60–414 fragments, or similarly related fragments (13, 20–23). Although the initial products retain...
activity in Pg activation, proteolysis at a number of other sites results ultimately in inactivation of SK. Intrinsically differences in the affinity of SK for Pg and Pm species accompanying these Pm-catalyzed reactions may contribute to the wide variation in estimates of the affinities of SK-Pg interactions from equilibrium binding studies. Binding studies using primarily surface-immobilized proteins have reported widely disparate dissociation constants for SK binding to (Glu) Pg ranging over ~10,000-fold, from 28 to 220,000 pm. A similarly undefined range of values have been reported for Pm, and these studies found equivalent affinity of SK for Pg and Pm (10, 13, 19, 24–27).

Fluorescent Pg analogs that are inactivated and covalently labeled at the zymogen catalytic site with probes were developed in previous studies to allow characterization of equilibrium binding interactions of SK in solution and without proteolysis occurring (19). In the present studies, this approach was extended to quantitatively characterize the interaction of SK with Pm under such conditions for the first time. Analysis of the fluorescence responses of an array of 16 active site-labeled fluorescent Pm derivatives shows that SK binds with an affinity that is 1,000–10,000 higher than that determined previously for Pg (19). This finding supports the novel conclusion that SK conformationally activates Pg in part by stabilization of the active conformation of the zymogen. Stoichiometric binding of SK to native Pm is followed by appearance of SK due to enhanced susceptibility of bound SK to cleavage by Pm. Comparison of the affinities of Pm for native SK, SK9, and a recombinant truncation mutant of SK reveals that cleavage at Lys29 alone has little effect on Pm binding, whereas deletion of the amino-terminal 54 residues decreases the affinity greatly. Kinetic studies revealed that the emergence of macromolecular substrate specificity for Pg activation which accompanied binding of SK to Pm was surprisingly linked to an overall decrease in specificity for tripeptide-p-nitroanilide (pNA) substrates. Binding of Pg as a substrate of the SK-Pm complex was directly demonstrated to be due to expression of a substrate recognition exosite on the SK-Pm complex. The results support the conclusion that SK binding to Pm induces changes in the catalytic site affecting the substrate-binding subsites, but that these changes are not responsible for the enhanced specificity for activation of Pg, and that an exosite expressed on the SK-Pm complex mediates SK-induced recognition of Pg as a specific substrate.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Characterization—**[Glu]Pg carbohydrate form 2 was purified from human plasma (28). Bovine Pg was purchased from Hematologic Technologies. Pm was prepared by activation of 10 μM [Glu]Pg with 90 units/ml urokinase (Calbiochem) in 10 mM Mes, 10 mM Hepes, 0.15 M NaCl, 20 mM 6-AHA, 1 mg/ml PEG, pH 7.4, at 25 °C, and isolated by affinity chromatography on soybean trypsin inhibitor-agarose by the published method (19, 29). Purified Pm was dialyzed against 50 mM Hepes, 0.3 M NaCl, 10 mM 6-AHA, 1 mg/ml PEG, pH 7.0, at 4 °C, and stored at <70 °C. Native SK was obtained from Diapharma and purified by affinity chromatography on a column (1.5 × 9 cm) of Pm immobilized onto iodoacetyl-(3,3′-iminobispropylamine)-agarose through the thiol group of the inhibitor. The proteins were expressed in Escherichia coli BL21(DE3)pLysS and immobilized in log phase at 50% ammonium sulfate to 50% saturation and mixing at 4 °C for 30 min. The precipitated was collected by centrifugation, dissolved in buffer containing the above inhibitors, and the precipitation was repeated twice. SK species were purified by affinity chromatography.

Protein concentrations were determined by absorbance at 280 nm using the following absorption coefficients (nm/mg* cm−1): and molecular weights; SK, 0.95 and 47,000 (31, 32); SK9, 1,49 and 47,000; SK55–414, 0.93 and 40,800; [Glu]Pg, 1.69 and 92,000; Pm, 1.9 and 84,000 (28). The absorption coefficients for Pm and the SK derivatives were determined from the amino acid composition and the measured absorbances in 0.1 M Hepes, 1 mM EDTA, ± 6 μg guanidine, pH 7.0 (33). Pm preparations were 75–90% active, as determined by titration with p-nitrophenyl p-guanidinobenzoate or fluorescein mono-p-guanidinobenzoate (34, 35).

**Gel Electrophoresis—**SDS-polyacrylamide gradient gels (Bio-Rad) were stained with GELCODE Blue Stain (Pierce) or SYPRO Orange fluorescent protein stain (Molecular Probes). For studies of SK cleavage by Pm, reactions were initiated at 25 °C by addition of Pm and stopped at various times by addition of 30 μM FFR-CH2Cl before preparation of the gel samples.

**Preparation of Active Site-labeled Pm—**ATA-FFR-CH2Cl and ATA-FCHR-CH2Cl were prepared as described previously (36–38). ATA-FFr-Pm and ATA-FFP-Pm were prepared by incubation of a 5-fold excess of inhibitor with 10–15 μM Pm in 0.1 mM Hepes, 0.3 M NaCl, 1 mM EDTA, 10 mM 6-AHA, 1 mg/ml PEG, pH 7.0, at 25 °C for 30–60 min, until inhibition was complete (>99.9%). Excess inhibitor was removed by dialysis against >250 volumes of 50 mM Hepes, 1 mM EDTA, pH 7.0, at 4 °C. Quantitation of the incorporation of ATA-FFFR-CH2Cl and ATA-FCHR-CH2Cl from the NH2-terminated burst of thiol measured with 5,5′-dithiobis(2-nitrobenzoic acid) (38) gave stoichiometries of 0.8–1.4 mol of thioester/mol of Pm active sites, with no significant free thiol (4 ± 5%) detected. FFP-Pm was prepared by reaction of Pm with FCHR-CH2Cl under the conditions given above.

For labeling with fluorescein probes, 0.8–1.2 ml reactions of 5–10 μM ATA-FFP-Pm or ATA-FFR-Pm and 70–120 μM of one of eight thiol-reactive probes were initiated by addition of 0.1 M NH4OH and incubated for 1 h at 25 °C in the dark. Excess dye was removed by chromatography on 9-ml columns of Sephadex G-25 (superfine), followed by incubation in the dark against >2000 volumes of the buffer used for preparations. The concentrations of the labeled probe derivatives were determined by absorbance or by micro-bicinchoninic acid protein assay (Pierce) with FFR-Pm as the standard. The concentrations of incorporated probes for ACR, badan, BODIPY, OG, and RhX were determined from the absorbance in 0.1 M Tris-Cl, 1 mM EDTA, 6 μg guanidine, pH 8.5. Absorption coefficients were determined by comparison of the free label absorbance in the guanidine buffer with the absorbance under the conditions used to determine previously published values (39). The resulting absorption coefficients (nm−1 cm−1) and wavelengths (nm) were: badan, 12.9 and 387; BODIPY, 63.6 and 508; OG, 55.8 and 491; ACR, 15.0 and 391; and RhX, 58.1 and 570. Incorporation of fluorescein and AANS were quantitated using the previously determined coefficients (39). Stoichiometries of probe incorporation were 0.7–1.5 mol of probe/mol of Pm active sites for all of the derivatives. The derivatives were homogeneous by SDS-gel electrophoresis, with the exception of faint bands corresponding to unlabeled, disulfide-bonded dimeric products (38).

**Fluorescence Studies—**Fluorescence measurements were made with an SLM 8100 spectrophotometer in the ratio mode, using acryl cuvettes coated with polyethylene glycol 20,000. Fluorescence titrations were performed at the excitation and emission maxima for the Pm derivatives, which were determined from spectra (4 nm band pass) in the absence and presence of 5 μM SK. The excitation and emission wavelengths (nm) used in titrations (8 or 16 nm band pass) were: AANS, 332, 456; aeryldan, 396, 502; badan, 399, 502; BODIPY, 513, 525; 5-F and 6-F, 500, 516; OG, 492, 517; and RhX, 578, 601. These values were identical for the ATA-FFR-CH2Cl and ATA-
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FPR-CH₂Cl derivatives, except for [AANS]FPR-Pm (332 nm excitation, 442 nm emission), and [RhX]FPR-Pm (578 nm excitation, 598 nm emission). Fluorescence titrations were performed by sequential addition of small volumes of SK to labeled Pm in 50 mM Hepes, 0.125 M NaCl, 1 mM EDTA, 1 mM PEG, 1 mg/ml bovine serum albumin, 1 μM FPR-CH₂Cl, pH 7.4, at 25 °C. Fluorescence changes were measured after equilibration for 5–10 min, and the measurements were expressed as the fractional change in the initial fluorescence (∆Fₐₕₒ/ₙₐₜ/Fₐₕₒ) = ∆F/Fₒ. Fluorescence changes were corrected for background by subtraction of measurements made on blanks lacking the labeled protein. For titrations in which labeled Pm derivatives were screened for SK-induced fluorescence changes, concentrations of labeled Pm were 50 μM to 82 nm, which were the lowest concentrations that could be used for each label to give background signals of <20%. Titrations were analyzed by nonlinear least-squares fitting of the quadratic binding equation, with the maximum fluorescence change (ΔFₚₑₐₓₜ/ₙₐₜ/Fₐₕₒ), dissociation constant (Kₛ), and stoichiometric factor (n) as the fitted parameters.

Measurements of the effect of native Pm on SK binding to [5-F]FPR-Pm were made following single additions of SK to cuvettes containing 75 μM [5-F]FPR-Pm and constant levels of native Pm, after a fixed incubation time of 10 min. The titrations were fit simultaneously by the equation for competitive binding of SK to native and labeled Pm to obtain the two dissociation constants, the stoichiometric factor for native Pm binding, and the maximum fluorescence change (40, 41).

Chromogenic Substrate Kinetics—The effect of SK on the kinetic parameters for hydrolysis of tripeptide-pNA substrates by Pm was measured from the initial rates (<5% substrate depletion) at 405 nm and 25 °C in 50 mM Hepes, 0.125 M NaCl, 1 mM EDTA, 0.125 M PEG, pH 7.4, in polyethylene glycol 20,000-coated cuvettes. All of the substrates were obtained from commercial sources except PGR-pNA which was synthesized by SynPept (Dublin, CA) and was 98% pure. Concentrations of peptide-pNA substrates were determined from the absorbance at 342 nm using an absorption coefficient of 8,266 M⁻¹ cm⁻¹, and product concentrations were calculated using an absorption coefficient of 9,933 M⁻¹ cm⁻¹ (35, 42). Reactions were initiated by addition of 0.25–100 nM Pm to buffer containing substrate in the absence or presence of a saturating concentration of SK (100–500 nM). The results were fit by the Michaelis-Menten equation to obtain Kₛ and kₗₛ. Substrate concentrations ranged up to 2 mM, representing 0.3–19 × Kₛ.

The difference in Gibbs free energy between the transition state for a substrate containing a single amino acid substitution compared with that of a reference substrate was calculated as,

\[ \Delta G_{\text{mbs}} = -RT \ln \left( \frac{s}{s_{\text{ref}}} \right) \]  

(Eq. 1)

where s and s_ref are the specificity constants (kₗₛ/Kₛ) for hydrolysis of the substrate and the reference substrate, respectively. \( \Delta G_{\text{mbs}} \) was calculated by subtraction of \( \Delta G_{\text{mbs}} \) in the absence of SK from that in the presence of saturating SK. The Gibbs free energy for the SK-induced change in specificity for a single substrate (\( \Delta G_{\text{SK}} \)) was calculated with Equation 2.

\[ \Delta G_{\text{SK}} = -RT \ln \left( \frac{s_{\text{SK}}}{s} \right) \]  

(Eq. 2)

where s and s_SK are the specificity constants in the absence and presence of saturating SK, respectively.

Initial rates of pyro-EPFP-pNA hydrolysis as a function of Pm, SK, and substrate concentrations were analyzed in a model with which SK acts as a hyperbolic mixed-type modifier of Pm activity (Scheme 1; Ref. 49).

\[ \begin{align*}
\text{Pm} + S & \xrightleftharpoons{K_{b}} \text{Pm} \cdot S \\
\text{SK} & \xrightarrow{K_{o}} \text{SK} \\
\text{SK} \cdot 
\end{align*} \]

\[ \begin{align*}
\text{Pm} \cdot S + S & \xrightarrow{K_{b}} \text{Pm} \cdot S \\
\text{SK} \cdot S & \xrightarrow{K_{o}} \text{SK} \cdot S \\
\text{SK} \cdot 
\end{align*} \]

\[ \text{Scheme 1} \]

In this model, Pm and the SK-Pm complex bind substrate (S) with dissociation constants, Kᵣ and aKᵣ, which are equal to Kᵣ and aKᵣ, and generate product (P) with catalytic rate constants, kₗₛ and βkₗₛ. SK binds to Pm with dissociation constant, Kₛ, and to the Pm-substrate complex (PmS) with dissociation constant, aKₛ. The dependence of the initial velocity (vₒ) on the total concentrations of substrate ([S]₀), the active Pm concentration ([Pm]₀), and the free SK concentration ([SK]₀) is given by Equation 3,

\[ v_{\text{obs}} = \frac{k_{\text{cat}}[\text{Pm}][S]_{0} + \beta k_{\text{cat}}[\text{S}]_{0}[\text{SK}]_{0}}{K_{m} + [S]_{0} + [\text{SK}]_{0} + \frac{[\text{S}]_{0}[\text{SK}]_{0}}{aK_{S}K_{D}}} \]  

(Eq. 3)

Because the assumption that [SK]₀ = [SK] was not valid in the case of a tight binding modifier under the conditions of the experiments, the results were analyzed by fitting of Equation 3, with the free SK concentration calculated with Equations 4 and 5,

\[ [\text{SK}]_{0} = [\text{SK} - \text{Pm}] - [\text{SK} \cdot \text{Pm} \cdot S] \]  

(Eq. 4)

\[ [\text{SK} + \text{Pm}] + [\text{SK} \cdot \text{Pm} \cdot S] = \frac{1}{2} \left( \frac{\alpha_{K_{P}} + a[S]}{\alpha_{K_{P}} - [S]} \right) + [\text{SK} + a[Pm]] - \left( \frac{\frac{\alpha_{K_{P}} + a[S]}{\alpha_{K_{P}} - [S]} + [SK] + a[Pm]}{\frac{aK_{P} + a[S]}{[S]} - aK_{P}} \right) \]  

(Eq. 5)

Equations 3–5 were algebraically equivalent to that developed by Szedenecsk et al. (45) for a tight-binding hyperbolic mixed-type inhibitor. The data collected as a function of SK and substrate concentrations were expressed as vₒ/[Pm]₀, and fit globally by Equations 3–5 to obtain Kₛ, aKₛ, α, and β, with the stoichiometric factor for SK binding fixed at 1.3 mol of SK/mol of Pm.

Plasminogen Activation Kinetics—The initial rate of bovine Pg activation by mixtures of SK and Pm was measured by continuous monitoring of the increase in absorbance of Tos-GPR-pNA at 405 nm (λₐₜ₉ₜₜ). The parabolic progress curves of pNA formation were fit by Equation 6 (8, 15),

\[ \frac{(\alpha_{K_{P}} + a[S])}{\alpha_{K_{P}} - [S]} = \frac{v_{\text{p}}^{2}}{2} + v_{\text{p}} + \frac{(A_{405} \text{nm})_{0}}{\epsilon} \]  

(Eq. 6)

where ε is the absorption coefficient for pNA. This analysis gave the initial rate of chromogenic substrate hydrolysis at the beginning of the reaction (vₒ) due to the Pm added initially, and the rate of the increase in activity with time due to bovine Pm formation (vₜ). The dependence of vₒ on the total PGP concentration ([BPg]₀) and the concentration of the SK-Pm complex ([SK-Pm]) under conditions of low chromogenic substrate concentration was given by Equation 7,

\[ v_{\text{p}} = \frac{k_{\text{BPg}}}{K_{\text{BPg}}} [S]_{0} \frac{k_{\text{BPg}}[\text{Pm}]_{0}}{K_{\text{BPg}} + [\text{BPg}]_{0}} \]  

(Eq. 7)

kₗₛ/Kₛₜₜₜ is the bimolecular rate constant for hydrolysis of the chromogenic substrate by bovine Pm, kₗₛₜₜₜ and Kₗₛₜₜₜ are the catalytic rate constant and Kₛ for bovine Pg activation, respectively. Bovine Pg activation by SK alone was subtracted from the results, and was <40% of the rate in the presence of Pm. The initial velocities measured as a function of [BPg]₀ at 0.5 mM Pm, 50 mM SK, and 100 μM D-Val-Leu-Lys-pNA were analyzed with Equations 3–5 and 7 to obtain the kinetic constants for bovine Pm activation by SK-Pm. Kₛₜₜₜₜ was not corrected for the small effect of competitive binding of the chromogenic substrate present at 0.3 aKₛ. The remaining kinetic constants were fixed at their determined values. To simplify the model further for analysis of the effect of SK binding to Pm on activation of BPg, experimental conditions were chosen with [S]₀ = 0.05 aKₛ, [S]₀ = 0.2 Kₛ, and [BPg]₀ = 100 mM ≪ Kₛₜₜₜₜ such that the first-order conditions for BPg activation were verified by the linear dependence of vₒ on BPg concentration up to 250 nM. Under these reaction conditions, the dependence of vₒ on SK concentration was given by the equations for the mixed modifier model described above, and the initial rate of the increase in activity due to BPg formation catalyzed by the SK-Pm complex is given by Equation 8,

\[ v_{\text{p}} = \frac{k_{\text{BPg}}}{K_{\text{BPg}}} [S]_{0} \frac{k_{\text{BPg}}[\text{Pm}]_{0}}{K_{\text{BPg}} + [\text{BPg}]_{0}} \]  

(Eq. 8)

where kₗₛₜₜₜ/Kₛₜₜₜₜ is the bimolecular rate constant for bovine Pm activation by SK alone was <3%
of the rate in the presence of Pm and was therefore neglected in the analysis. The initial velocities measured as a function of the total SK concentration at 0.1 nM Pm and 25 or 50 μM chromogenic substrate were analyzed with Equations 3–5 and 8 to obtain the dissociation constant for SK binding to native Pm and the bimolecular rate constant for BPg activation by SK-Pm. The remaining kinetic constants were fixed at their determined values.

Least-squares fitting was performed with Scientist software (MicroMath). Uncertainties in reported parameters are ±2 S.D. Experimental error in the kinetic parameters was propagated.

RESULTS

Screening of Active Site-labeled Fluorescent Derivatives of Plasmin as Probes of Streptokinase Binding—To identify fluorescent derivatives of Pm for characterizing SK binding, an array of 16 derivatives was prepared by inactivation of Pm with ATA-FPR-CH2Cl or ATA-FFR-CH2Cl and labeling the thiol group generated on the amino terminus of the inhibitor with each of eight thiol-reactive probes (36, 37). Fluorescence excitation and emission spectra in the absence and presence of saturating (5 μM) SK showed that all labeled Pm species except [BODIPY]FPR-Pm and [RhX]FFR-Pm reported perturbations of the active site environment with significant changes in fluorescence of 20–107%. No significant changes (±3 nm) in excitation and emission spectral maxima were observed, with the exception of [AANS]FFR-Pm, for which SK produced a 7-nm red shift in the emission spectrum. Analysis of the fluorescence titrations showed maximum enhancements of 27–107% for the polarity-sensitive probes, AANS, ACR, and badan (Fig. 1A), whereas the fluorescence of Pm labeled with either of two structural isomers of fluorescein, OG or RhX, was quenched 14–49% (Fig. 1B). The amplitudes of the fluorescence changes for three of the probes, ACR, BODIPY (Fig. 1A), and RhX (Fig. 1B) were affected significantly (2–9-fold) by the substitution of Phe for Pro in the P2 (nomenclature of Schechter and Berger (44)) position of the linking inhibitor peptide.

Characterization of SK Binding to [5-F]FFR-Pm—Analysis of the titrations in Fig. 1 indicated that SK bound with very high affinity, which precluded an accurate estimation of the dissociation constants for several of the derivatives labeled with less intensely fluorescent probes. Single titrations of the four fluorescein and one RhX derivatives, which could be done at low Pm concentrations (65–170 pm), yielded indistinguishable dissociation constants ranging from 5 ± 7 pm to 15 ± 9 pm (Fig. 1). The average stoichiometric factor determined from all of the titrations was 1.1 ± 0.5 mol of probe/mol of Pm. On the basis of the high quantum yield of fluorescein and the large fluorescence change reported by [5-F]FFR-Pm on binding SK, this derivative was chosen for further studies of SK binding. Characterization of the Pm derivative confirmed that it was stoichiometrically labeled (1.1 ± 0.1 mol of probe/mol of active sites) and that the probe was specifically incorporated into the catalytic site-containing light chain (Fig. 2, inset). Simultaneous fitting of SK titrations of [5-F]FFR-Pm at fixed concentrations of 75 pm, 1 nm, and 10 nm by the equation for a single binding interaction gave a dissociation constant of 11 ± 2 pm, a stoichiometric factor of 1.3 ± 0.1 mol of SK/mol of Pm, and a maximum fluorescence change of −48 ± 1% (Fig. 2).

Proteolytic Cleavage of SK by Native Pm—To characterize the interaction of native Pm with SK, it was first necessary to evaluate proteolytic cleavage of SK by Pm. Incubation of 250 nm SK with an excess of Pm resulted in complete disappearance of native SK from 4 to 15% SDS gradient gels within 10 min and appearance of a new band with an 8,000 lower apparent molecular weight (Fig. 3). Results of similar experiments with 10–20% gradient gels showed that the disappearance of native SK, which migrated with an apparent molecular weight of 49,000, was correlated with appearance of two new bands at apparent molecular weights of 44,000 and 6,000 (results not shown). The SK cleavage product was assigned to the noncovalent complex between SK1–59 and SK60–414 (SK) or a closely related secondary species on the basis of the apparent molecular weights and the results of previous studies identifying this and similar modified forms of SK (20–22).

Reactions of a fixed concentration of SK with increasing concentrations of Pm in the 0.1–1 μM range showed quantitative conversion of SK to SK’ at equimolar levels of Pm and SK, and in the presence of up to a 4-fold excess of Pm (Fig. 3). When SK was present in excess, however, additional proteolysis products were observed (Fig. 3, A and B) that appeared to be the result of slower cleavage of free SK by the SK-Pm complex, on the basis that these were the predominant species present. To investigate this further, the effect of active site-blocked Pm (FFR-Pm) on cleavage of SK was examined. In reactions containing a 4-fold excess of SK over active Pm but no FFR-Pm, formation of SK’ and the additional incomplete cleavage products were observed as before (Fig. 3C). When sufficient FFR-Pm was present to bind essentially all of the SK, the heterogeneous products were not observed and all of the SK was converted to SK’ within 2 min (Fig. 3C). These results indicated that SK bound to Pm or FFR-Pm was preferentially cleaved at an enhanced rate by free Pm and the SK-Pm complex. Similar results were obtained at 20–40 nm Pm and SK, the lowest concentrations that could be studied (results not shown).

Binding of SK to Native Pm—The above results indicated that SK complexes with native Pm were only transiently stable at ≥20 nm concentration. To characterize the binding of SK to native Pm and the possible effect of SK cleavage, [5-F]FFR-Pm was used as a probe to estimate the affinity of the initially formed complexes between native Pm and SK. The fluorescence of [5-F]FFR-Pm was stable (±3%) in the absence and presence of up to 1 nm native Pm for at least 40 min. Following addition of SK to mixtures of native and labeled Pm, the fluorescence decreased to a stable value within ±5 min and increased gradually ≥10% over the following 20–30 min. The steady state formed in the first 10 min was taken to represent equilibration of initially formed SK complexes with native and labeled Pm. Titrations of [5-F]FFR-Pm with SK in the absence and presence of 0.1, 0.2, and 1 nm native Pm (Fig. 4) were fit well by the equation for competitive binding of SK to native and labeled Pm, with a dissociation constant of 12 ± 4 pm for SK binding to 1.3 ± 0.3 sites on native Pm and an equivalent value of 16 ± 4 pm for SK binding to [5-F]FFR-Pm (Fig. 4).

Binding of SK’ and a Truncated SK Mutant to [5-F]FFR-Pm—To examine further the influence of SK’ formation on its interaction with Pm, binding of SK’ and a recombinant SK mutant lacking the amino-terminal 54 (SK(55–414)) residues were examined. The SK noncovalent complex was purified from a reaction mixture of Pm and SK, and contained both major fragments of SK’, along with smaller amounts of other degradation products evident by SDS-gel electrophoresis (Fig. 5, inset). SK’ bound to labeled Pm with a dissociation constant of 43 ± 1 pm and maximum fluorescence change of −44 ± 1%, while wild-type recombinant SK bound with a dissociation constant of 33 ± 6 pm and a 49 ± 1% change (Fig. 5). Although these dissociation constants were 3–4-fold larger than the value of 12 ± 4 pm obtained for native SK and Pm, they were considered indistinguishable because of the uncertainty inherent in accurate estimation of parameters for such high affinities, and the presence of some degradation products in the SK’ preparation. By contrast, the purified SK truncation mutant, SK(55–414) bound [5-F]FFR-Pm with a dissociation constant of 4 ± 1 nm, representing a loss of 120–360 fold in affinity (Fig. 5).
Fig. 1. Fluorescence titrations of an array of fluorescent Pm derivatives with SK. The fractional change in fluorescence ($\Delta F/F_0$) is plotted versus the ratio of the total concentrations of SK to Pm ([SK]/[Pm]) for 16 fluorescent derivatives of Pm, prepared by inactivation with either ATA-FPR-CH$_2$Cl or ATA-FFR-CH$_2$Cl, as indicated by ATA-FPR-Pm and ATA-FFR-Pm at the top, and labeling with each of the thiol-reactive probes indicated on the right. Those Pm derivatives which showed an enhancement in fluorescence are shown in A, while those which exhibited a quench are in B. The lines represent the least squares fits of the binding equation, with the indicated fitted values of the maximum fluorescence change (%) and the dissociation constants for those derivatives that could be studied at low enough concentration to obtain reliable values. Fluorescence titrations were performed and analyzed as described under “Experimental Procedures.”
Moreover, SK$^{55-414}$ produced a 3-fold smaller fluorescence change of 16 ± 1% compared with native SK, indicating a significant difference in the perturbations of the active site (Fig. 5). These results indicated that the cleaved, noncovalent SK' complex had nearly the same properties as native SK with respect to Pm interactions, whereas deletion of the 54 residues in SK$^{55-414}$ resulted in a large loss of affinity for Pm.

Effect of SK on the Specificity of Pm for Tripeptide Chromogenic Substrates—Michaelis-Menten kinetic parameters were determined for Pm and the SK-Pm complex for an array of 11 tripeptide-pNA substrates. For 8 of the substrates, significant decreases of 2.6–10-fold in catalytic specificity ($k_{cat}/K_m$) of Pm were observed upon SK binding, while 3 substrates were unaffected or decreased by less, and only CBO-RGR-pNA was increased, by 1.8-fold (Table I). The overall decrease in specificity was generally attributable to increases of 2–11-fold in $K_m$, with smaller changes of $-2$-fold in $k_{cat}$. PGR-pNA, which has the sequence corresponding to the activation site in Pg cleaved specifically by SK-Pm was the poorest of the tripeptide substrates examined and the specificity of Pm for this substrate was reduced 2.6-fold by SK binding (Table I). The free energy differences calculated from the ratio of the specificity constants for Pm and SK-Pm ($\Delta G_{SK}$) demonstrated that SK binding reduced the stability of the transition state by up to 1.4 kcal/mol, depending on the structure of the substrate (Table II).

The possible contribution of SK' formation to the observed changes in chromogenic substrate activity was investigated in extensive control experiments comparing the order of addition of the reactants and the effect of preincubation of SK and Pm on the rates. All of the reactions were linear over the 5–20-min duration of the assays, and the rates of pyro-EPR-pNA hydrolysis varied less than ±7% for assays initiated with Pm or with substrate, over a range of Pm (0.1–10 nM), SK (0.1–100 nM), and substrate (50–600 μM) concentrations. The rates of reactions initiated with chromogenic substrate were similarly stable (±7%) over 20–30 min of preincubation of SK and Pm. These results indicated that the changes in activity occurred rapidly on mixing SK and Pm and did not change significantly over the time course of the experiments. If cleavage of SK occurred during these experiments, it did not measurably affect the properties of the complexes with Pm.

To evaluate the relationship between SK binding and the specificity of Pm for the P1-P3 positions in the substrates, D-VLK-pNA, Tos-GPR-pNA, and PGR-pNA were chosen as reference substrates for comparison of free energy changes accompanying single residue substitutions (46, 47). $\Delta G_{subs}$ was calculated for each substrate, which represented the difference in free energy of the transition state for the substrate and the reference substrate (Table II). The difference between $\Delta G_{subs}$ for Pm and SK-Pm ($\Delta \Delta G_{subs}$) represents the free energy for the SK-induced change in transition state stability relative to the reference substrate. Comparison of single substitutions of Arg for Lys at P1 in D-VLK-pNA and Lys for Arg in Tos-GPR-pNA showed little difference in the free energy change induced by SK, as given by $\Delta \Delta G_{subs}$, indicating little change in the preference of the S1 site as a result of SK binding (Table II). For Pm, favorable decreases in $\Delta G_{subs}$ were observed for a single substitution of Phe for Leu at P2 in D-VLK-pNA and for substitutions of both Phe at P2 and pyro-E at P3, whereas these effects were significantly less favorable for SK-Pm, demonstrating a differential effect of SK binding on the S2 subsite. Although substitutions at P3 resulted in significant changes in specificity, these were generally paralleled by Pm and SK-Pm, demonstrating little differential effect of SK binding (Table II).
SK on hydrolysis of pyro-EPR-pNA, studied as a function of SK and substrate concentrations at 0.1 nM Pm (Fig. 6). The combined results were fit well by the mechanism with a dissociation constant of 11 ± 5 pm for SK binding to native Pm, and kinetic constants that were indistinguishable from those determined in the previous experiments (Fig. 6 and Table 1). These results demonstrated the quantitative correspondence between high affinity SK binding to native Pm and its linkage with the decrease in specificity for peptide-pNA substrates.

Effect of SK Binding to Pm on Bovine Pg Activation—The relationship between SK binding to Pm and specificity for Pg activation was examined in kinetic studies of bovine Pg activation. Advantage was taken of the fact that bovine Pg is activated only very poorly by SK alone, but, like human Pg, is a specific substrate of the SK-Pm complex (7–9). For these experiments, the parabolic progress of pNA formation for reaction mixtures of Pm, SK, Tos-GPR-pNA, and BPg were analyzed to determine the initial rate of substrate hydrolysis at the beginning of the reaction due to the Pm present at zero time (vᵢ), and the rate of bovine plasmin formation (vᵢ) from the increase in activity with time. Low concentrations of chromogenic substrate and BPg were chosen to simplify the analysis (see “Experimental Procedures”). Under these conditions, the rate of Tos-GPR-pNA hydrolysis catalyzed by Pm (vᵢ) decreased to a non-zero limiting value in titrations with SK (Fig. 7A), as predicted from the inhibitory effect of SK as a mixed modifier of Pm activity with this substrate (Table 1). The rate of BPg activation in the same reactions was undetectable in the absence of SK, and increased to a saturating value with SK concentration that represented an estimated >50-fold enhancement in the rate. Under the conditions used, the SK saturation curves in Fig. 7B represented the binding of SK to human Pm, independent of the chromogenic substrate and BPg concentrations (see “Experimental Procedures”). Analysis of the results in this manner gave a dissociation constant of 23 ± 9 pm for SK binding and a bimolecular rate constant for activation of BPg at saturating SK of 8.4 ± 0.3 × 10⁻⁵ M⁻¹ s⁻¹ (Fig. 7B). These results demonstrated directly the correspondence between high affinity binding of SK to Pm, the loss of specificity for the chromogenic substrate, and the simultaneous expression of macromolecular substrate specificity for Pg.

Direct Evidence for Exosite-mediated Substrate Recognition of BPg by the SK-Pm Complex—Binding of BPg to [AANS]FPR-Pm was examined in the absence and presence of excess SK to evaluate the contribution of exosite interactions to BPg substrate recognition induced by SK binding to Pm. SK bound stoichiometrically to [AANS]FPR-Pm and enhanced the fluorescence maximally 84 ± 1%. Subsequent titration with BPg produced a saturable further increase in the fluorescence of [AANS]FPR-Pm of 92 ± 24% in the presence of saturating levels of SK (Fig. 8A). In the absence of SK, BPg produced no significant change in fluorescence (<3%, Fig. 8B) indicating no interaction of BPg with labeled Pm alone. Analysis of the results gave a dissociation constant of 2.9 ± 1.3 μM for BPg binding to the SK-[AANS]FPR-Pm complex. This value was independent of the presence of SK in excess of Pm (Fig. 8A) and indistinguishable from the Kₘ determined under these conditions for BPg activation by the native SK-Pm complex of 3.6 ± 1.3 μM (results not shown). These results demonstrate that high affinity binding of SK to Pm is accompanied by expression of a substrate-binding exosite for BPg. This interaction must occur through an exosite because the S1-S3 specificity sites are blocked in [AANS]FPR-Pm by the fluorescent label. Exosite-mediated binding is principally responsible for BPg substrate recognition.
Effect of streptokinase binding to plasmin on hydrolysis of tripeptide-pNA substrates

| Substrate       | Pm, $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | SK · Pm, $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|-----------------|--------------|-------|---------------|-------------------|-------|---------------|
|                 | s⁻¹          | μM    | µM⁻¹ s⁻¹ × 10⁻³ |                   |       |               |
| d-Val-pNA       | 16.9 ± 0.5   | 140 ± 10 | 120 ± 10 | 34 ± 2 | 300 ± 50 | 120 ± 10 |
| d-Val-pNA       | 21.6 ± 0.8   | 310 ± 30 | 70 ± 7   | 38 ± 2 | 600 ± 50 | 63 ± 6   |
| d-Val-pNA       | 9.9 ± 0.5    | 9.6 ± 0.5 | 1030 ± 70 | 17.4 ± 0.5 | 56 ± 5 | 310 ± 30 |
| Pyro-EF-pNA     | 60.7 ± 0.8   | 52 ± 3 | 1160 ± 80 | 36.8 ± 0.7 | 150 ± 10 | 240 ± 20 |
| Tos-GPR-pNA     | 50 ± 1       | 250 ± 20 | 200 ± 20 | 44 ± 3 | 1000 ± 100 | 44 ± 6 |
| Tos-GPF-pNA     | 52 ± 1       | 130 ± 30 | 380 ± 30 | 26 ± 2 | 480 ± 80 | 50 ± 10 |
| Pyro-EPR-pNA    | 69 ± 3       | 100 ± 100 | 700 ± 100 | 87 ± 4 | 390 ± 40 | 220 ± 30 |
| d-Ipr-pNA       | 27 ± 1       | 310 ± 30 | 90 ± 10 | 31 ± 2 | 3500 ± 400 | 8.7 ± 0.8 |
| PGR-pNA         | 3 ± 2        | 4200 ± 2700 | 0.7 ± 6  | 1.2 ± 0.3 | 4100 ± 1400 | 0.3 ± 0.1 |
| CBO-RGR-pNA     | 6.8 ± 0.4    | 180 ± 30 | 38 ± 7   | 8.4 ± 0.2 | 120 ± 10 | 70 ± 6   |
| EGR-pNA         | 36 ± 10      | 7000 ± 2000 | 5 ± 2   | 18 ± 2 | 9000 ± 2000 | 1.9 ± 0.4 |
| GR-pNA          | 0.24 ± 0.05  | 10000 ± 3000 | 0.024 ± 0.009 | 0.11 ± 0.03 | 7000 ± 3000 | 0.016 ± 0.008 |

**Table I** Thermodynamic analysis of the effect of streptokinase binding on the specificity of plasmin for tripeptide-pNA substrates

The free energy differences in the stability of the transition states accompanying substitutions at P1–P3 in the reference substrates are listed as $ΔG_{subs}$. Values were calculated from the specificity constants listed in Table I. The differences in free energy between Pm and SK · Pm for the substitutions in the reference substrates are listed as $ΔG_{subs}$. The free energy for the SK-induced change in specificity for each substrate is listed as $ΔG_{SK}$. Substitutions in reference substrate are indicated in bold. Experiments were performed and thermodynamic parameters were calculated as described under "Experimental Procedures."

| Substrate       | P3 | P2 | P1 | $ΔG_{subs}$ | $ΔG_{subs}$ | $ΔG_{subs}$ | kcal/mol |
|-----------------|----|----|----|-------------|-------------|-------------|
| Pyro-E          | F  | K  | K  | −1.3 ± 0.2  | −0.4 ± 0.1  | 0.9 ± 0.2   | 0.9 ± 0.1 |
| Tos-G           | P  | R  | R  | −0.4 ± 0.1  | −0.1 ± 0.1  | 0.3 ± 0.1   | 1.2 ± 0.2 |
| Pyro-E          | P  | R  | R  | −0.8 ± 0.1  | −1.0 ± 0.2  | −0.2 ± 0.2  | 0.7 ± 0.1 |
| d-Ipr           | P  | R  | R  | 0.5 ± 0.1   | 1.0 ± 0.2   | 0.5 ± 0.2   | 1.4 ± 0.2 |
| P               | G  | R  | R  | −2.3 ± 2.0  | −3.3 ± 1.4  | −0.9 ± 2.4  | −0.4 ± 0.1 |
| CBO-R           | G  | R  | R  | −1.2 ± 1.1  | −1.1 ± 0.5  | 0.0 ± 1.2   | 0.6 ± 0.3 |
| E               | G  | R  | R  | 2.0 ± 1.8   | 1.7 ± 1.1   | −0.3 ± 2.1  | 0.3 ± 0.2 |

**DISCUSSION**

Active site-selective fluorescence labeling of Pm with thioester peptide chloromethyl ketones enabled quantitative characterization of SK-Pm binding interactions in solution and allowed the binding interactions to be resolved from coupled proteolytic activation and degradative reactions for the first time. The results show that SK binds with high affinity to active site-labeled fluorescent Pm derivatives under conditions where proteolysis does not occur, and induces a change in the Pm active site environment reported by a diverse array of fluorescent probes. Binding of SK to Pm is quantitatively correlated with an unexpected decrease in specificity of native Pm for peptide-chromogenic substrates, simultaneous emergence of macromolecular specificity for activation of Pg through expression of a specific substrate recognition exosite, and enhanced Pm-catalyzed cleavage of SK. In previous equilibrium binding studies with fluorescent analogs of Pm, dissociation constants of 590 ± 110 nM were determined for [AAN]FFR-[Glu]Pg, 11 ± 7 nM for [AAN]FFR-[Lys]Pg, and 90 ± 60 nM for SK binding to native [Glu]Pg (19). Comparison of these results with the dissociation constant of 12 ± 4 µM found here for Pm indicates that SK exhibits a dramatic, 1,000–10,000-fold higher affinity for Pm compared with the Pg zymogen. The observation of indistinguishable dissociation constants for SK binding to four fluorescein and one rhodamine derivatives of Pm demonstrated that the affinity for SK was not significantly perturbed by the presence of the labels in the active site. Moreover, use of [5-F]FFR-Pm as a probe of competitive SK binding to native Pm yielded an indistinguishable dissociation constant. It should also be recognized, however, that there is disagreement among published studies concerning the dissociation constants for SK binding to Pg and Pm, which vary widely and some differ substantially from those reported here (10, 13, 24–27). The dissociation constant of 50 µM inferred from the kinetic effect of SK binding on Pm inactivation by streptantiplasmin (10) is in agreement with the present direct binding studies. A potentially significant methodological difference that could contribute to the discrepancy in affinities is that surface-immobilized proteins were used primarily in other previous studies, whereas the present studies and that of Cederholm-Williams et al. (10) characterized the interactions in solution.

The SK-induced changes in fluorescence of the Pm derivatives may signal a conformational change affecting the microenvironment of the probes in the active site, and/or close proximity of SK to the probes in the complex. The variation of the probe responses to SK binding is thought to reflect changes in substrate specificity that affect the subsites occupied by the probe-tripeptide label, in combination with the individual re-
Binding of Streptokinase to Plasmin

Fig. 6. Effect of SK binding on the kinetics of Pm-catalyzed hydrolysis of pyro-EPR-pNA. A, dependence of the initial rate divided by the enzyme concentration ($v_{cat}$/[Pm]) as a function of substrate concentration ([pyro-EPR-pNA]) was measured at 0.25–0.5 mM Pm in the absence (○) and presence (○) of 100 mM SK. B, dependence of the rate on the total SK concentration ([SK]) measured at 0.1 mM Pm in the presence of 50 μM (●), 250 μM (●), 500 μM (●), and 2000 μM (●) pyro-EPR-pNA. Solid lines in both panels represent the global nonlinear least-squares fit of the data with the mixed-type modifier model, as described under “Experimental Procedures.” The fitted parameters for binding of SK were: $K_d = 11 \pm 5$ μM and $αK_d = 40 \pm 20$ μM. Kinetic parameters for substrate hydrolysis by Pm were $k_{cat} = 101 \pm 8$ μM and $k_{cat} = 69 \pm 1$ s$^{-1}$, and for the SK-Pm complex were $k_{cat} = 430 \pm 50$ μM and $βk_{cat} = 90 \pm 3$ s$^{-1}$. Experiments were performed and analyzed as described under “Experimental Procedures.”

Fig. 7. Effect of SK binding to Pm on bovine Pg activation. Activation reactions containing 0.1 mM Pm, 100 mM BPg, 25 or 50 μM Tos-GPR-pNA, and various SK concentrations were resolved into the rate of chromogenic substrate hydrolysis due to the Pm added ($v_1$) and the rate of BPg formation ($v_2$) as described under “Experimental Procedures.” A, dependence of $v_1$ on the total SK concentration ([SK]) in the presence of 50 μM Tos-GPR-pNA. B, dependence of the rate of BPg activation ($v_2$) on SK concentration in the presence of 25 μM (●) or 50 μM (●) Tos-GPR-pNA. The solid lines in B represent the least-squares fit of the data with the dissociation constant for SK binding to Pm and the maximum rate of BPg activation given in the text. The solid line in A represents the curve calculated from the fitted and independently determined parameters. Reactions were performed and analyzed as described under “Experimental Procedures.”

SK binding are thought to reflect the initially less structured environment of the Pm active site, and possibly more homogeneous changes induced by SK.

Analysis of the effect of SK on chromogenic substrate hydrolysis by Pm supported a mechanism in which SK acts as a tight binding mixed-modifier of Pm activity, binding to native Pm with the same dissociation constant as obtained in the fluorescence studies. Positive ΔGSK values for 11 of 12 peptide substrates that were due mainly to increases in $K_m$ indicated that substrate binding to the active site opposes the change induced by SK for many substrates. This opposition may be steric because SK binds in the vicinity of the active site (11), but more likely represents a conformational change in Pm that reduces the affinity for substrate. Although this effect also predicts the likelihood of negative linkage between SK affinity and occupation of the active site by the peptide-probe fluorescent labels, this effect was evidently small for the Pm derivatives studied here. Examination of the differences in the stability of the transition states for pairs of Pm substrates accompanying the substitution of P1-P3 residues demonstrated the effects of SK on subsite specificity. Pm exhibits a slight preference for Lys over Arg at P1 (47), whereas the opposite specificity would presumably be optimal for cleavage of the Arg561-Val562 bond required for Pg activation by the SK-Pm complex. The specificity of S1 for Arg or Lys, however, was not affected by SK. Substitutions at P2 had a differential effect on Pm and SK-Pm, corroborating the fluorescence results and indicating that the S2 specificity of free Pm and Pm bound to SK were uniquely
Stoichiometric binding of SK to native Pm was coupled with enhanced formation of SK cleaved at Lys\(^{59}\) by Pm and SK-Pm, possibly due to a conformational change in SK which made this site more susceptible to cleavage. A number of approaches were taken to evaluate the influence of this proteolytic reaction on the interaction of SK with Pm. Purified preparations of SK' bound with similar affinity to [5-F]FFR-Pm, produced a fluorescence change of nearly the same amplitude as native SK, and had an effect similar to native SK on the chromogenic substrate activity of Pm. These results indicated little effect of the Lys\(^{59}\) cleavage on the binding affinity, the change in active site environment, or substrate specificity changes. By contrast, a deletion mutant of SK in which 54 residues were removed from the amino terminus showed 120–360-fold reduced affinity for Pm, raising the dissociation constant to a still significant value of 4 ± 1 nM. This observation supports the conclusion that interactions of SK\(^{1–59}\) and SK\(^{60–414}\) within the SK' complex and with Pm contribute substantially to the affinity of binding. Interestingly, interactions of the amino-terminal region of SK also play a role in perturbing the properties of the catalytic site, as evidenced by the large decrease in the amplitude of the fluorescence change for the SK\(^{55–414}\) mutant. An incompletely resolved issue concerning SK' is the residual uncertainty about whether the results of the fluorescence and kinetic studies with native Pm reflect native SK, or SK' generated by Pm proteolysis. The mechanism of SK' formation appears to be through cleavage of SK bound to Pm by free Pm or by the SK-Pm complex, suggesting that at the low concentrations used in these experiments this process may have been slow, and the results may reflect the properties of native SK. While this is uncertain, the results of comparison of SK and SK' indicate that they have indistinguishable characteristics with respect to the properties studied here.

The large increase in affinity for SK accompanying the conversion of Pg to Pm provides new insight into the mechanism of conformational activation of Pg. The structure of SK bound to the catalytic domain of Pm shows that the SK γ-domain makes critical contacts with part of the 142–152 segment of the chymotrypsinogen-homologous serine proteinase activation domain, in addition to interactions with other loops (11, 52). The activation domain of chymotrypsinogen-likezymogens consists of the four segments 16–19, 142–152, 184–193, and 216–223, which are disordered in thezymogen and undergo folding to form the active enzyme conformation (52). The activating conformational change is triggered by proteolytic generation of the new amino terminus at Ile\(^{16}\), and its insertion into the amino-terminal binding pocket (52, 53). The energy cost of folding of the Pg sequence homologous to the 142–152 segment during the conversion of Pg to Pm may thus contribute to the higher affinity of SK for the active enzyme. Pg and other serine proteinasezymogens are thought to be in pre-existing equilibrium between inactive and active conformations (52, 53) with equilibrium constants favoring the inactive conformation by 10\(^3\) for trypsinogen (54) to about 12 for single chain tissue-type plasminogen activator (55). The Pgzymogen has ~10\(^{-7}\)-fold lower activity than Pm, indicating that the finding of a comparable, 10\(^3\)-10\(^4\)-fold increase in affinity of SK for Pm suggests that SK conformationally activates Pg in part by stabilizing the activated conformation of thezymogen. Further studies will be needed to determine whether the conformational change is induced by SK binding to Pg, and/or is the result of shifting the pre-existing unfavorable equilibrium toward the activezymogen conformation. The preferential affinity of SK for Pm also indicates that in the overall mechanism of activation of Pg by SK, the product of the reaction (Pm) binds to SK more tightly than Pg. It is speculated that this large increase in affinity...
plays an essential role in the catalytic mechanism by trapping the stable activated SK-Pm complex, which then recognizes Pg as a specific substrate through expression of the Pg-binding exosite, and proteolytically converts Pg to Pm.

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Streptokinase Binds to Human Plasmin with High Affinity, Perturbs the Plasmin Active Site, and Induces Expression of a Substrate Recognition Exosite for Plasminogen

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