Rapid Binding of Plasminogen to Streptokinase in a Catalytic Complex Reveals a Three-step Mechanism*

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Background: We previously showed that plasmin binding to streptokinase is a three-step mechanism with a slow off-rate. Rapid kinetics demonstrate a three-step pathway of streptokinase (SK) binding to plasminogen (Pg), the zymogen of plasmin (Pm). Formation of a fluorescently silent encounter complex is followed by two conformational tightening steps reported by fluorescence quenches. Forward reactions were defined by time courses of biphasic quenching during complex formation between SK or its COOH-terminal Lys414 deletion mutant (SKΔK414) and active site-labeled [Lys]Pg ([5-(acetamido)fluorescein]-d-Phe-Phe-Arg-[Lys]Pg ([5F]FFR-[Lys]Pg)) and by the SK dependences of the quench rates. Active site-blocked Pm rapidly displaced [5F]FFR-[Lys]Pg from the complex. The encounter and final SK-[5F]FFR-[Lys]Pg complexes were weakened similarly by SK Lys414 deletion and blocking of Ile1-Ala2 residues of SK into the binding cleft of the Pg protease domain to form Pm (6, 8–14). Conformational activation of Pg in the catalytic SK-Pg* complex by the molecular sexuality mechanism involves insertion of the NH2-terminal Ile1-Ala2 residues of SK into the binding cleft of the Pg protease domain (9, 11, 12, 15–17). Ile1 binds Pg Asp194 (chymotrypsin numbering), causing expression of the substrate-binding site and formation of the oxyanion hole (15, 16, 18, 19). The mechanism is also valid for conformational prothrombin activation by staphylocoagulase and von Willebrand factor-binding protein from Staphylococcus aureus (20, 21). This mechanism allows group A and C streptococci to hijack Pg in the human fibrinolytic system by quorum sensing-induced secretion of SK. This results in localized plasmin generation for dissolution of host fibrin barriers and facilitated bacterial spreading (22–25).

In our unified model, the conformationally activated SK-Pg* complex binds Pg as a substrate and cleaves it to Pm. This is the

The serine proteinase plasmin (Pm)2 is primarily known for its role in dissolving fibrin thrombi (1). It also causes cell surface remodeling, signaling, and cancer progression (2). Proteolytic activation of the zymogen plasminogen (Pg) by tissue plasminogen activator and urokinase-type plasminogen activator differs from conformational activation by the non-enzymatic streptococcal pathogenicity factor streptokinase (SK). We studied SK from Streptococcus dysgalactiae subsp. equisimilis because of its 90% homology with phylogenetic cluster 1SKs from the human host-specific, virulent Streptococcus pyogenes (3). S. dysgalactiae subsp. equisimilis, which is generally opportunistic in horses, also causes severe human infections such as bacteremia, pneumonia, endocarditis, arthritis, and streptococcal toxic shock syndrome (4, 5).

The Pg activation mechanism by SK is unique (6–8). Stoichiometric binding of SK to Pg and Pm forms catalytically active SK-Pg* and SK-Pm complexes that bind Pg as a substrate in SK-Pg* complex and cleave Arg561-Val562 in the Pg protease domain to form Pm (6, 8–14). Conformational activation of Pg in the catalytic SK-Pg* complex by the molecular sexuality mechanism involves insertion of the NH2-terminal Ile1-Ala2 residues of SK into the binding cleft of the Pg protease domain (9, 11, 12, 15–17). Ile1 binds Pg Asp194 (chymotrypsin numbering), causing expression of the substrate-binding site and formation of the oxyanion hole (15, 16, 18, 19). The mechanism is also valid for conformational prothrombin activation by staphylocoagulase and von Willebrand factor-binding protein from Staphylococcus aureus (20, 21). This mechanism allows group A and C streptococci to hijack Pg in the human fibrinolytic system by quorum sensing-induced secretion of SK. This results in localized plasmin generation for dissolution of host fibrin barriers and facilitated bacterial spreading (22–25).
Streptokinase-Plasminogen Binding Pathway

EXPERIMENTAL PROCEDURES

Protein Purification and Characterization—[Glu]Pg carbohydride form 2 was purified from human plasma and activated to [Lys]Pg and [Lys]Pm (Pm) as described (8, 26, 27, 60, 61). Pm was purified by affinity chromatography on soybean trypsin inhibitor-agarose and dialyzed against 5 mM HEPES, 0.3 M NaCl, 10 mM 6-AHA, 1 mg/ml PEG 8000, pH 7.0 at 4 °C. The active Pm concentration (~90%) was determined by titration with fluorescein mono-p-guanidinobenzoate (62).

Proteins were quick frozen in 2-propanol/dry ice and stored at ~80 °C. Protein concentrations were determined by absorbance at 280 nm using the following absorption coefficients (mg/ml)^−1 cm^−1 and molecular weights: [Glu]Pg, 1.69 and 92,000; [Lys]Pg, 1.69 and 84,000; Pm, 1.9 and 84,000 (47, 61, 64); SK and SKΔK414, 0.81 and 47,000 (65, 66); SKΔ(K253–L260)ΔK414-His_6, 0.78 and 49,213 (63).

Active Site Labeling of Pg—[Glu]Pg and [Lys]Pg were labeled at the active site as described previously (27, 58, 63). The SKΔ(K253–L260)ΔK414-His_6, mutant activates [Lys]Pg conformationally, but the complex does not readily cleave Pg to Pm, and the use of this SK construct for Pg labeling significantly increased the yield of labeled Pg and reduced the preparation time (63). Labeled Pg concentration and probe incorporation (~90%) were determined from the probe and protein absorbances in 6 M guanidine as described (48, 54, 55). Proteins were homogeneous by SDS gel electrophoresis.
**Streptokinase-Plasminogen Binding Pathway**

Stopped-flow Kinetics of nSK, WT-SK, and SKΔK414 Binding to [5F]FFR-Pg—Complete progress curves of SK binding to labeled Pg were captured with an Applied Photophysics SX-18MV stopped-flow spectrophotometer in single mixing mode with excitation at 500 nm and an emission cut-off filter (Melles-Griot) with 50% transmission at 515 nm. Changes in fluorescence intensity were measured for all the reactions, and for the interaction of native SK with [5F]FFR-[Lys]Pg in the absence of lysine analogs, changes in fluorescence anisotropy were also monitored. The reaction volume was 200 μl, the path length was 2 mm, and experiments were performed at 25 °C. Binding was studied under pseudo-first-order conditions (SK length was 2 mm, and experiments were performed at 25 °C in buffer systems. Binding of WT-SK (0.050–10 mM 6-AHA or in 50 mM HEPES, 0.075 M NaCl, 1 mM EDTA, 1 mg/ml PEG 8000, 1 mg/ml bovine serum albumin, 1 μM FFR-CH2Cl, pH 7.4 in the absence and presence of 50 mM 6-AHA or in 50 mM HEPES, 0.075 M NaCl, 1 mM EDTA, 1 mg/ml PEG 8000, 1 mg/ml bovine serum albumin, 1 μM FFR-CH2Cl, pH 7.4 containing 50 mM benzamidine to maintain constant ionic strength. Binding of nSK and SKΔK414 (0.050–18 μM) to [5F]FFR-[Lys]Pg (10–20 nm) was studied in all three buffer systems. Binding of WT-SK (0.050–10 μM) to [5F]FFR-[Lys]Pg (20 nm) in the absence of effectors was included as a control. Binding of native SK (0.1–6 μM) to [5F]FFR-[Glu]Pg (13 and 20 nm) was studied in the absence and presence of 50 mM 6-AHA. Averaged time traces (1,000 data points/trace and 10 traces for each SK concentration) of the decrease in fluorescence intensity or increase in anisotropy ranged from 0.4 to 50 s, depending on the SK concentration and the presence of effector. Averaged time traces of blank titrations containing buffer and SK only and buffer and [5F]FFR-Pg only were obtained to measure background light scattering and initial probe fluorescence, respectively, and to permit transformation of raw data into the fractional change in initial fluorescence ((Fobs - F0)/F0 = ΔF/F0) or anisotropy ((robs - r0)/r0 = Δr/r0). Subtracting background scattering was critical as the signal-to-noise ratio for reactions with [5F]FFR-Pg (~25% quench and ~7.5% maximal scattering) was up to 5-fold smaller than that for reactions with [5F]FFR-Pm (~50% quench and ~6% maximal scattering) (59). Experiments were limited to labeled Pg concentrations up to 20 nm due to the lower solubility of the SK-Pg complex compared with that of SK-Pm, which resulted in a substantial increase in background scattering at Pg concentrations above ~40 nm. None of our previously published studies of SK binding and kinetics have used [Lys]Pg and [Glu]Pg concentrations exceeding 20 and 30 nm, respectively. Averaged time traces were analyzed using Equation 1.

\[
\Delta F/F_0 = \frac{(F_0 - F_0)(A_1 e^{-k_{obs1} t} + (1 - A_1) e^{-k_{obs2} t}) + F_M}{(A_1 e^{-k_{obs1} t} + (1 - A_1) e^{-k_{obs2} t}) + F_M}
\]  

(Eq. 1)

where \(F_0\), is the starting fluorescence, \(F_M\) is the final fluorescence, \(A_1\) is the fractional amplitude of the fast exponential component, \(1 - A_1\) is the fractional amplitude of the slow exponential component, and \(k_{obs1}\) and \(k_{obs2}\) are the observed first-order rate constants for the fast and the slow conformational changes. The rate constants were analyzed as a function of the total SK concentration ([SK]_t) using Equation 2.

\[
k_{obs1,2} = \frac{k_{lim1,2} [SK]_0}{k_1 + [SK]_0 + k_{off1,2}}
\]  

(Eq. 2)

where \(K_i\) is the dissociation constant for the SK-Pg encounter complex and \(k_{lim1,2}\) and \(k_{off1,2}\) are the limiting rates and the reverse rate constants for each conformational step, respectively.

**Competitive Dissociation of [5F]FFR-[Lys]Pg from its Complex with nSK, WT-SK, and SKΔK414 by FFR-Pm**—In stopped-flow experiments, [5F]FFR-[Lys]Pg and SK or SKΔK414 were preincubated in the dark for 5 min at 25 °C and loaded in one syringe. FFR-Pm was loaded in the second syringe, and time traces of fluorescence increase for the reverse reactions were recorded until displacement was >90% complete, ranging from 15 to 200 s. Final concentrations in the cell at the dead time of mixing: [5F]FFR-[Lys]Pg, 10–20 nm; SK or SKΔK414, 59–2,000 nM; and FFR-Pm, 100–2,000 nm. Background scattering was subtracted, and \(F_o\) values of free [5F]FFR-[Lys]Pg were established, i.e., the signal at 100% displacement. Fluorescence quenches of the preformed SK-[5F]FFR-[Lys]Pg complexes in the dead time (4 ms) of the mixing step with FFR-Pm were compared for consistency with the values for forward reactions of SK and [5F]FFR-[Lys]Pg under identical conditions. Unlike the SK reactions with labeled Pg, the reactions with Pg were not stoichiometric due to weaker SK binding, and a range of SK and FFR-Pm concentrations was used to obtain time traces at various degrees of SK saturation with labeled [Lys]Pg and FFR-Pm. The faster and much larger displacement signal for FFR-Pm compared with FFR-Pg and the vastly lower scattering background of the SK-FFR-Pm complex were major reasons for performing displacement experiments with FFR-Pm rather than with FFR-Pg. The time traces were fit by a double exponential function (analogous to Equation 1) to obtain the observed first-order rate constants \(k_{disp1}\) and \(k_{disp2}\) for the fast and slow displacement processes.

**Equilibrium Binding of [5F]FFR-Pg to SK and SKΔK414 in the Presence of Benzamidine—[5F]FFR-Pg (10 nm) was titrated with SK or SKΔK414 at 25 °C in 50 mM HEPES, 0.075 M NaCl, 1 mM EDTA, 1 mg/ml PEG 8000 buffer, pH 7.4 containing 50 mM benzamidine, 1 mg/ml BSA, and 1 μM FFR-CH2Cl. Fluorescence titrations were performed with a Photon Technology International, Inc. fluorometer at excitation and emission wavelengths of 500 and 516 nm, respectively, with 2/8-nm excitation/ emission band passes. Fluorescence changes were measured after equilibration for 5–10 min. Measurements were corrected for background (~10%) by subtraction of blanks lacking [5F]FFR-Pg. Data were analyzed by the quadratic equation for binding of a single ligand (55). This analysis gave the dissociation constant (\(K_d\)) for binding of SK or SKΔK414 to [5F]FFR-Pg and the maximum fluorescence intensity change (\(ΔF_{max}/F_0\)) with a stoichiometric factor (n) of 1 for binding of SK or SKΔK414 to labeled Pg.

Two-exponential time traces of forward and reverse reactions, SK dependences of \(k_{obs1,2}\) and equilibrium binding of SK and SKΔK414 to [5F]FFR-Pg in benzamidine buffer were analyzed by nonlinear least square fitting with SCIENTIST Software (MicroMath). All reported estimates of error represent ±2 S.D.
Numerical Integration Analysis of the Forward and Reverse Reactions—Arrays of progress curves for SK-[5F]FFR-Pg formation and displacement of labeled Pg from the complex were analyzed globally with the numerical integration program KinTek Explorer 3.0 (67–69) for each set of reactants, concentration ranges, and buffer conditions. Five arrays were performed in 6-AHA: fluorescence amplitude changes of [5F]FFR-[Lys]Pg binding to nSK, WT-SK, and SKΔK414; fluorescence amplitude changes of [5F]FFR-[Glu]Pg binding to nSK; and anisotropy changes of [5F]FFR-[Lys]Pg binding to nSK. Three arrays were performed in 6-AHA: fluorescence amplitude changes of [5F]FFR-[Lys]Pg binding to nSK and SKΔK414 and [5F]FFR-[Glu]Pg binding to nSK. Two arrays were performed in benzamidine: fluorescence amplitude changes of [5F]FFR-[Lys]Pg binding to nSK and SKΔK414.

The mechanism included Scheme 1 for three-step SK-[5F]FFR-Pg binding. The dissociation constants $K_1$ and $K_2$ for formation of the SK-Pg 1 and SK-Pm 1 encounter complexes represent the ratios $k_{-1}/k_1$ and $k_{-4}/k_4$ where $k_1$ and $k_4$ are the second-order association rate constants and $k_{-1}$ and $k_{-4}$ are the first-order rate constants for dissociation of the encounter complex. $K_1$, $k_2$, $k_{-2}$, $k_3$, and $k_{-3}$ are equivalent to $K_1$, $k_{lim}$, $k_{off}$, $k_{lim}$, and $k_{off}$ respectively, in Equation 2. The three-step mechanism for SK-Pm stabilization was validated in a previous study (59).

Time traces of fluorescence quenches were transformed to increases by plotting $ΔF/Δt$ expressed as functions of the formation and stabilization of SK-Pg 1, SK-Pg 2, and SK-Pg 3 complexes using positive amplitude factors as KinTek Explorer does. The overall change in fluorescence intensity or anisotropy was expressed as $ΔF/Δt = f_2 × ([SK-Pg 2]/[Pg]_o) + f_3 × ([SK-Pg 3]/[Pg]_o)$, where $[Pg]_o$ is the total [5F]FFR-Pg concentration, which is equal to the sum of $P_{free}$, SK-Pg 1, SK-Pg 2, and SK-Pg 3; $[SK-Pg 2]$ and $[SK-Pg 3]$ are the concentrations of these complexes at time $t$; and $f_2$ and $f_3$ are the respective fractional amplitude factors for these complexes. The SK-Pg 1 complex does not contribute to fluorescence change. This expression allowed simultaneous analysis of time traces with different [5F]FFR-Pg concentrations.

Fitting Strategy—The on-rate constant $k_1$ for formation of the encounter complex was initially constrained at $1 \times 10^8 \text{M}^{-1} \text{s}^{-1}$ as determined experimentally for SK binding to unlabeled [Lys]Pg (7), and the assumption was made that on-rates would apply for reactions of SK with [5F]FFR-[Lys]Pg and [5F]FFR-[Glu]Pg and of SKΔK414 with [5F]FFR-[Lys]Pg in all of our experimental buffers. Upon refinement of the other parameters, fitting $k_1$ yielded values that were close to $1 \times 10^8 \text{M}^{-1} \text{s}^{-1}$ under all these conditions, justifying our choice of this value as an initial estimate. The parameters $K_1$, $k_2$, $k_{-2}$, $k_3$, and $k_{-3}$ were initially constrained to $K_1$, $k_{lim}$, $k_{off}$, $k_{lim}$, and $k_{off}$ obtained from the SK dependences (Table 1, superset b), and refinement of these initial estimates ultimately provided the final fits (superscripts a and aa).

Analysis of [5F]FFR-[Lys]Pg displacement required known concentrations of free Pg and the intermediates SK-Pg 1, SK-Pg 2, and SK-Pg 3 present at the start of the reaction with FFR-Pm as there was substantial partitioning among these species at equilibrium of the SK-[5F]FFR-[Lys]Pg complex. They were calculated iteratively using the starting concentrations of SK and [5F]FFR-[Lys]Pg used to form the complex, the forward and reverse rate constants, and the known dissociation constant for the competitive, unlabeled SK-FFR-Pm complex. The sum of the calculated free Pg, SK-Pg 1, SK-Pg 2, and SK-Pg 3 concentrations was in agreement with the total Pg concentration, indicating that mass balance was conserved during the fits. Complexes of SK with labeled and unlabeled Pg have indistinguishable affinities in the absence of lysine analogs (26, 48) and in 6-AHA (26, 27), suggesting that the binding parameters for SK are very similar for labeled and unlabeled FFR-Pm. This allowed fixing $K_2$, $k_{-2}$, $k_3$, and $k_{-3}$ to our previously determined values for [5F]FFR-Pm binding in each buffer system (59). Displacement of [5F]FFR-[Lys]Pg binding to SK and SKΔK414 in benzamidine was analyzed with fitted $K_2$, values of $227 \pm 11$ and $200 \pm 20 \text{pm}$ for FFR-Pm binding, respectively, in agreement with the previously determined $130$ and $250 \text{pm}$ (59).

The large scattering background introduced variable uncertainty in the amplitude factors $f_2$ and $f_3$ for [SK-Pg 2] and [SK-Pg 3] at increasing SK concentrations, resulting in non-random residuals when imposing global $f_2$ and $f_3$ fits on the complete data sets. Initial estimates of the rate constants obtained by global fitting of $f_2$ and $f_3$ were fixed, and individual $f_2$ and $f_3$ amplitude factors were assigned as fitted parameters for time traces at each SK concentration. This largely eliminated the non-random deviations. Subsequent fixing of all the individual amplitude parameters provided further refinement of the fitted rate constants with only subtle differences from the original estimates.

The overall $K_D$ values for the final, stabilized complexes were calculated from Equation 3 using the rate constants obtained by numerical analysis and compared with $K_D$, obtained independently from equilibrium binding.

$$K_D = \frac{[SK][Pg]}{[SK-Pg 1] + [SK-Pg 2] + [SK-Pg 3]}$$

$$= \frac{k_1K_2K_3}{1 + K_1K_2 + K_3} \quad \text{(Eq. 3)}$$

RESULTS

Stopped-flow Kinetics of SK Binding to [5F]FFR-[Lys]Pg—Time traces of fractional quenches of fluorescence intensity ($ΔF/Δt$) and increases of anisotropy ($Δr/r_o$) following rapid mix-
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FIGURE 1. Stopped-flow fluorescence changes of SK binding to [5F]FFR-[Lys]Pg. A and B, the fractional fluorescence intensity changes (∆F/F₀) following rapid mixing of [5F]FFR-[Lys]Pg and nSK versus time are shown in the absence of lysine analogs at 20 nM [5F]FFR-[Lys]Pg and 0.42, 0.84, 1.58, 3.00, and 5.00 μM nSK (A) and at 10 nM [5F]FFR-[Lys]Pg and 8.2, 11.7, and 17.6 μM nSK (B). C and D, fractional fluorescence anisotropy changes (∆rₐ) are shown for 20 nM [5F]FFR-[Lys]Pg and 0.075, 0.15, 0.20, and 0.52 μM nSK (C) and for 4.7, 7.0, and 14 μM nSK (D). Green and blue solid lines represent the fits from numerical integration with the parameters given in Table 1 as described under “Experimental Procedures.”

FIGURE 2. SK concentration dependence of the kinetics of [5F]FFR-[Lys]Pg binding in the absence of lysine analogs. Dependences of k₁obs and k₂obs on [SK]₀ are shown for binding to 10–20 nM [5F]FFR-[Lys]Pg. The inset shows the k₁obs dependence on an enlarged scale. Solid and dashed lines represent the least square fits by Equation 2 with the parameters given in Table 1 for the reactions with nSK and WT-SK, respectively.

ing of [5F]FFR-Pg with excess SK in the absence of lysine analogs were distinctly biexponential with first-order rate constants k₁obs and k₂obs fitted by Equation 1. Time traces started at zero ∆F/F₀ with F at 4 ms F₀ of a control reaction with only [5F]FFR-Pg, indicating no significant fluorescence change associated with encounter complex formation. Representative changes in fluorescence intensity and anisotropy of [5F]FFR-[Lys]Pg binding to nSK are shown in Fig. 1. Colored lines represent global fits of forward and reverse reactions by numerical analysis. The first-order rate constants k₁obs and k₂obs for the fast and slow fluorescence changes obtained from the individual biexponential fits increased hyperbolically with increasing SK concentration. Fig. 2 shows the nSK and WT-SK dependences in the absence of effectors. The hyperbolic dependences of k₁obs and the much smaller k₂obs, respectively, indicated saturation of the encounter complex and the subsequent conformational intermediate (Fig. 2, inset). The parameters Kᵣ, k₁lim, k₂lim, k₁off, k₂off, and k₉off determined by fitting the binding rate constants by Equation 2 are given in Table 1 (superscript b). The reverse rate constants k₁off and k₂off for the two conformational steps given by the extrapolated intercepts of k₁obs and k₂obs at zero SK were 4.0 ± 1.0 and 0.25 ± 0.10 s⁻¹. The dissociation constants Kᵣ for the encounter complexes of [5F]FFR-[Lys]Pg with nSK and WT-SK were 3.4 ± 1.0 and 2.1 ± 1.2 μM.

Stopped-flow Kinetics of SKΔK414 Binding to [5F]FFR-[Lys]Pg—Progress curves of SKΔK414 binding to [5F]FFR-[Lys]Pg and the SKΔK414 dependence of k₁obs and k₂obs are shown in Fig. 3 with colored lines representing the global fits of the forward and reverse reactions by numerical analysis. The SKΔK414 mutant bound ~6-fold more weakly than nSK with a Kᵣ of 19 ± 4 μM (Table 1, superscript b), suggesting that the COOH-terminal SK Lys₁⁴⁻硃 increases the efficiency of initial docking of SK with Pg by interacting with a kringle.

Effects of 6-AHA on the Kinetics of SK and SKΔK414 Binding to [5F]FFR-[Lys]Pg—Time traces of the forward reactions and the SK dependences of k₁obs and k₂obs in 50 mM 6-AHA are shown in Fig. 4. Blocking the LBSs on kringles K1, K4, and K5 with 6-AHA decreased the affinity of the encounter complex to a Kᵣ value of 7 μM (Table 1, superscript b), which is comparable with that of SKΔK414 binding in the absence of 6-AHA. The SK and SKΔK414 dependences of these weak binding interactions were not saturable, preventing accurate determination of Kᵣ; hence values ranging from 7 to 20 μM may be considered comparable. A weak encounter complex with a Kᵣ value of 14 μM was also observed for SKΔK414 binding in 6-AHA (Fig. 5). The limiting rate constants k₁lim and k₂lim and the off-rates k₁off and k₂off determining the conformational changes following encounter complex formation were similar for SK and SKΔK414 binding to [5F]FFR-[Lys]Pg in the absence and presence of 6-AHA. Total amplitudes of the time traces fit by Equation 1 reflected overall maximal fluorescence changes (∆F₀max/F₀) for SK and SKΔK414 binding to [5F]FFR-Pg in agreement with equilibrium binding results in the absence and presence of 6-AHA (27, 48).

Effects of Benzamidine on the Kinetics of SK and SKΔK414 Binding to [5F]FFR-[Lys]Pg—Blocking kringles K1, K2, and K5 with 50 mM benzamidine weakened the Kᵣ of the [5F]FFR-[Lys]Pg encounter complex with SK to 12 μM (Table 1, superscript b). Time traces of the forward reactions of SK binding were not resolvable into two phases and appeared as single exponential curves, whereas SKΔK414 binding was clearly biphasic. Progress curves for binding of SK and SKΔK414 and their concentration dependences of k₁obs and k₂obs in benzamidine are shown in Fig. 6. The limiting rate k₁lim in benzamidine was ~5-fold faster for SK binding and ~2.3-fold faster for SKΔK414 binding compared with the values in 6-AHA and in the absence of lysine analogs (Table 1, superscript b). The ~30% lower maximal fluorescence changes than those for equilibrium binding in the presence of benzamidine described below (Table 1) may be due to the scattering properties of Pg complexes in benzamidine being differentially affected by the optical cell geometry and path length of the Photon Technology International, Inc. fluorometer and the stopped-flow instrument.
TABLE 1
Kinetic and equilibrium binding parameters for the formation of SK-Pg and SKΔK414-Pg complexes

Kinetic constants obtained from simultaneous numerical integration of the forward and reverse reactions, forward reactions measured as anisotropy changes, and SK dependences of the fast and slow phases of the forward reactions are listed for reaction Scheme 1 in the absence of kringle ligands (no effector) and in the presence of saturating 6-AHA or benzamide. $K_{D,overall}$ was calculated from the individual kinetic parameters measured by fluorescence titration. For analysis of near-linear SK dependences $k_1$ was fixed to the value obtained by numerical analysis. Amplitudes of change in fluorescence intensity following rapid mixing of [5F]-FFR-[Lys]Pg and SK represent the fits by Equation 2 with the parameters given in Table 1. Reported errors are $2 \times$ S.D. and were calculated by error propagation for compound parameters.

|                  | $K_1$ (nM) | $k_{ disappearance}$ | $k_{ formation}$ | $k_{ disappearance}$ | $k_{ formation}$ | $k_{ f,overall}$ | $\Delta F/F_0$ |
|------------------|------------|----------------------|------------------|----------------------|------------------|-----------------|---------------|
| [5F]FFR-[Lys]Pg, no effector nSK | 2.8 ± 0.3a | 34 ± 2a | 3.5 ± 0.4a | 0.34 ± 0.04a | 0.15 ± 0.01a | 0.086 ± 0.018ae | -30 ± 9a, -25 ± 6a |
| WT-SK | 1.8 ± 0.2ab | 33 ± 2a | 1.8 ± 0.1ab | 0.26 ± 0.06ab | 0.12 ± 0.02ab | 0.030 ± 0.018ab | -28 ± 1b |
| SKΔK414 | 3.4 ± 2b | 33 ± 2a | 4.0 ± 1b | 0.90 ± 0.3b | 0.25 ± 0.1b | 0.044 ± 0.009b | -26 ± 8, -24 ± 4 |
| 6-AHA nSK | 20.3 ± 0.2a | 31 ± 1a | 2.5 ± 0.1a | 0.17 ± 0.01a | 0.20 ± 0.01a | 0.84 ± 0.09ae | -24 ± 4, -30 ± 5 |
| SKΔK414 | 19.0 ± 4.0b | 25 ± 4a | 2.3 ± 0.6b | 0.10 ± 0.08b | 0.30 ± 0.06b | 0.60 ± 0.20a | -24 ± 2a |
| Benzamidine nSK | 11.4 ± 0.3a | 156 ± 37a | 3.2 ± 0.1a | 0.07 ± 0.01a | 0.15 ± 0.02a | 0.16 ± 0.04ae | -18 ± 7, -17 ± 8 |
| SKΔK414 | 12.0 ± 2.0a | 150 ± 15a | 2.0 ± 1.0a | 2.00 ± 0.20a | 0.19 ± 0.09a | 0.56 ± 0.09a | -31 ± 1a |
| 6-AHA nSK | 16.0 ± 0.3b | 69 ± 4a | 4.8 ± 0.2b | 0.26 ± 0.02b | 0.24 ± 0.02b | 0.52 ± 0.06ae | -27 ± 7, -23 ± 3 |
| SKΔK414 | 16.0 ± 10.0b | 70 ± 30b | 4.0 ± 2.0b | 1.30 ± 0.40b | 0.27 ± 0.10b | 0.80 ± 0.10a | -38 ± 1a |

Stopped-flow Kinetics of SK Binding to [5F]FFR-[Glu]Pg—Biexponential binding of SK to [5F]FFR-[Glu]Pg was not saturable, and the $k_2/K_1$ ratios in the absence and presence of 6-AHA were indistinguishable and similar to those for [5F]FFR-[Lys]Pg binding to SKΔK414 in the absence of kringle ligands and to nSK and SKΔK414 binding in 6-AHA (Fig. 7). Fitting of these near linear dependences was performed using fixed, lower limit $K_1$ values that were reasonably resolvable by numerical integration (see below). The limiting rate constants $k_{lim,1}$ and $k_{lim,2}$ and the off-rates $k_{off,1}$ and $k_{off,2}$ were similar to those for SK and SKΔK414 binding to [5F]FFR-[Lys]Pg in the absence of lysine analogs and in 6-AHA (Table 1, superscript b).

Competitive Displacement of [5F]FFR-[Lys]Pg from Its Complex with nSK, WT-SK, and SKΔK414 by FFR-Pm—Mixing a 1:1 molar ratio of unlabeled FFR-Pm with the preformed complexes of SK and SKΔK414 with [5F]FFR-[Lys]Pg at varying degrees of saturation caused a rapid, biexponential increase of fluorescence, approaching the initial fluorescence intensity. Analysis of the time traces by Equation 1 yielded perfect fits with random residuals (not shown) and gave $k_{disp,1}$ and $k_{disp,2}$ values for the fast and slow exponential phases of the displacement reactions. Representative averaged traces for SK and SKΔK414 displacement by FFR-Pm are shown in Figs. 8 and 9 with the colored lines representing global fits of forward and reverse reactions by numerical analysis. These processes represent rapid reversal of [5F]FFR-[Lys]Pg binding to SK and SKΔK414 and parallel formation of non-fluorescent complexes with FFR-Pm. Saturation of Pg required high SK and SKΔK414 concentrations and consequently high FFR-Pm to bind free SK and SKΔK414 and to displace labeled Pg from the complexes. Therefore we expressed $k_{disp,1}$ and $k_{disp,2}$ as dependences of free conditions.
rather than total FFR-Pm calculated by numerical integration.

The rates were independent of free FFR-Pm, consistent with extremely tight binding of SK and SKΔK414 to plasmin (59), and were similar for [Lys]Pg and [Glu]Pg in the absence and presence of lysine analogs. The off-rate for the fast process, $k_{\text{off1}}$, was $0.90 \pm 0.60 \text{ s}^{-1}$, which is modestly lower than the averaged $k_{\text{off1}}$ of $2.60 \pm 1.2 \text{ s}^{-1}$ determined from the SK dependences of the forward reactions and the equivalent averaged $k_{-2}$ of $3.2 \pm 1.6 \text{ s}^{-1}$ from numerical analysis (see below). The $k_{\text{disp2}}$ off-rate for the slow phase was $0.13 \pm 0.09 \text{ s}^{-1}$, which is similar to the averaged $k_{\text{disp2}}$ of $0.22 \pm 0.12 \text{ s}^{-1}$ from the SK dependences and the equivalent averaged $k_{-3}$ of $0.19 \pm 0.08 \text{ s}^{-1}$ from numerical analysis.

Equilibrium Binding of [5F]FFR-[Lys]Pg to SK and SKΔK414 in the Presence of 50 mM Benzamidine—We determined the affinity and fluorescence change for SK and SKΔK414 equilibrium binding to [5F]FFR-[Lys]Pg in 50 mM benzamidine to characterize the effect of this K1, K2, and K5 ligand on the overall equilibrium binding constant $K_{D,\text{overall}}$ and to compare this affinity with $K_{D,\text{overall}}$ calculated from the forward and reverse constants obtained by the binding kinetics (Fig. 10). Analysis of the titrations indicated that SK bound with a $K_D$ of $200 \pm 20 \text{ nM}$ and $\Delta F_{\text{max}}/F_0$ of $-38 \pm 1\%$ and $K_D$ of $800 \pm 100 \text{ nM}$. This affinity was similar to that of SK in 6-AHA, SKΔK414 with and without 6-AHA (48), and SK binding to [5F]FFR-[Glu]Pg with and without 6-AHA (27, 48).

Numerical Integration Analysis of the Forward and Reverse Reactions—Fitted values for $K_D$, the rate constants for both conformational steps, and the fluorescence amplitudes were in good agreement with those obtained from two-exponential analysis and equilibrium binding and are given in Table 1 (superscript a, fluorescence intensity, and superscript aa, fluorescence anisotropy). The results indicated that formation of fluorescently silent SK-Pg 1 occurs in the dead time of the reaction and that subsequent partitioning occurs between SK-Pg 2 and SK-Pg 3.

The SK-[5F]FFR-[Lys]Pg encounter complex was weakened $\sim$10–20-fold by blocking LBSs on the Pg kringles and by loss of Lys$^{\text{414}}$. The rate constant $k_2$ for the first conformational step ranged from 25 to 45 s$^{-1}$ in the absence and presence of 6-AHA.
but increased substantially in benzamidine, suggesting a decrease in conformational restraint.

The rate constants $k_{-2}$ and $k_{-3}$ for the reverse reactions were equivalent to $k_{off 1}$ and $k_{off 2}$ from hyperbolic fitting of the SK dependences of the forward reaction rates and to $k_{disp 1}$ and $k_{disp 2}$ for the bimolecular appearance of free [5F]FFR-[Lys]Pg in competitive displacement by FFR-Pm. The analytical solution of the overall $k_{off}$ value for a three-step reaction is only straightforward under conditions of single exponential kinetics (70); however, the agreement of $k_{off 1}$ and $k_{disp 2}$ with $k_{-3}$ from numerical analysis suggests that dissociation is limited by $k_{-3}$. The off-rates were unaffected by lysine analogs.

In the absence of effectors, $K_{D, overall}$ for SK binding to [5F]FFR-[Lys]Pg ranged from 30 ± 18 to 86 ± 18 nM in agreement with the results from equilibrium binding (27, 48). Deletion of SK Lys414 or blocking the LBSs with 6-AHA caused an increase of $K_{D, overall}$ to 0.5–0.9 μM, which is identical to that for [Glu]Pg binding. In benzamidine, $K_{D, overall}$ for binding of intact SK to labeled [Lys]Pg was 0.2 μM, possibly reflecting the contribution of the large forward rate for the first tightening step. Within global data sets, the errors in the amplitude factors $f_2$ for the fast conformational step and $f_1$ for the slow step were ~30 and ~18%, respectively (2 × S.D.). Numerical integration fits for the forward and reverse reactions are shown as colored lines in the figures.

**DISCUSSION**

The present study demonstrates a minimal three-step sequential mechanism for binding of SK to [5F]FFR-Pg, consisting of an encounter complex with affinity in the low micromolar range followed by at least two resolvable conformational
Lys-Pg are mainly limited to SK Lys414 binding to K4, whereas changes caused a 9,000-fold tightening of the Pm encounter complex but only a ~50-fold increase in affinity for Pg. We show here that substantial decreases in affinity of the encounter complex and the second conformational event are mainly responsible for the weaker SK binding to Pg in the stabilized complex.

The results suggest that the SK interactions with LBSs on [Lys]Pg are mainly limited to SK Lys414 binding to K4, whereas...
plasmin binding involves another SK lysine interacting with K5 in addition to Lys414 binding to K4. It is noteworthy that non-LBS interactions with the protease domain are significant sources of binding energy in both plasminogen and plasmin binding (71). Until now, SK binding to Pg had only been studied by equilibrium binding, and although the published values report the affinities of the final complexes, they do not provide information on the intermediates in this multistep mechanism.

The results support the following sequential steps on the pathway to a stabilized complex with labeled Pg: SK Lys414 binding to a Pg kringle during formation of a weak, fluorescently silent encounter complex and two conformational steps of SK reorganization from a flexible to a more organized form during binding to the Pg protease domain accompanied by expression of a pro-exosite for binding of a second Pg molecule in the substrate mode. This reorganization is reported by biphasic fluorescence changes of the probe in the active site on the protease domain of Pg. Two striking differences between SK binding to labeled Pm and [Lys]Pg were immediately obvious: a ~40-fold weaker binding of SK in the encounter complex illustrated by higher SK concentrations required for saturation of the rates of fluorescence change and the requirement of stopped flow to study Pg displacement from the complex by FFR-Pm evidenced by the large increase in the off-rate constants k-2 and k-3. Whereas displacement from the SK-Pm complex required several hours of incubation with excess FFR-Pm, the complexes with [Lys]Pg were easily reversed in a matter of seconds.

Binding to Pg also involves insertion of the NH2 terminus of SK in the activation pocket of the Pg catalytic domain; however, adding a conformational step to the mechanism did not improve the fits. Stopped-flow fluorescence of SK binding to labeled Pg may not allow identifying the timing of the NH2-terminal insertion or whether NH2-terminal insertion contributes to the affinity of the Pg complex, and further studies are required to resolve this complex event.

Binding of SK Lys414 to a kringle facilitates formation of the encounter complexes with both [Lys]Pg and Pm as a similar 6–8-fold reduction in their affinity was observed when Lys414 was deleted. K0 of the encounter complex with labeled [Lys]Pg increased from ~3 to ~19 μM upon deleting SK Lys414. Satura-
tion of the LBSs did not decrease the affinity of SK and SKΔK414 any further, indicating no other SK lysine-LBS interactions, and the 10–19 μM affinity range of LBS-blocked SK and SKΔK414 complexes likely represents the contribution of non-LBS binding to the Pg catalytic domain (Table 1). K0 of the encounter complex with Pm increases from ~0.08 to ~0.67 μM upon SK Lys414 deletion (59); however, the SKΔK414-Pm encounter complex still exhibits substantial affinity, reflecting the sum of the LBS interactions with other lysine residues and non-LBS interactions with the protease domain. Kringle K5 harbors an LBS that preferentially interacts with ligands not carrying a free carboxylate function, such as alkyamines (51, 72, 73), and K5 on Pm may bind a non-COOH-terminal SK lysine. Saturation of Pm with 6-AHA disengages Lys414 and other lysines, and as expected, this affinity is not weakened further by SK Lys414 deletion. The remaining encounter affinity of ~5–8 μM likely represents the non-LBS interactions with the Pm catalytic domain (59). Multiple LBS interactions in the tighter encounters complex with Pm may be made possible by an increased flexibility of two-chain Pm compared with single chain Pg. This flexibility might also allow more intimate contacts during stabilization of the SK-Pm complex.

6-AHA binds kringle K1, K4, and K5, and the Pg binding results likely eliminate the involvement of K2 and K3 in SK Lys414 binding. Similarly, the weak SK binding to [Glu]Pg eliminates K1 as a candidate for Lys414 interaction as this is the only kringle in [Glu]Pg exposed for fibrin binding (74). Kringle K4 is not accessible in [Glu]Pg due to steric hindrance by the Pg NH2-terminal PAN module, which binds K5 (75, 76). The identical encounter complex affinity of SK for [Glu]Pg in the absence and presence of 6-AHA indicated that LBS interactions do not play a role in [Glu]Pg binding.

Benzamidine blocks kringles K1, K2, and K5 and leaves kringle K4 available for lysine binding. The affinity of the SK-Pg encounter complex in 6-AHA and benzamidine was similar, suggesting that Lys414 binding to K4 in Pg does not increase the affinity when K5 is blocked. However, the SK-Pm encounter complex was ~2-fold tighter in benzamidine than that in 6-AHA and was further weakened by deletion of Lys414 (59). Further studies are required to clarify these different effects on Pg and Pm binding.

The 42-residue COOH-terminal sequence is not resolved in the SK-μPm crystal structure (19). Lys414 at the end of this disordered, mobile sequence may guide the pathway by initial interaction with the LBS on K4; however, this does not contribute much to the free energy of binding of the encounter complex. Calculating changes in free energy of association for SK-binding pathway.
and SKΔK414 binding to Pg and Pm from ΔG° = RT ln(K<sub>L</sub>) using averaged K<sub>L</sub> values from Table 1 and our previous study (59) shows that the non-LBS interactions contribute ~83 and ~73% of the binding energy in Pg and Pm encounter complex formation, respectively. SK Lys<sup>414</sup> contributes ~17 and ~13%. Binding of (an)other SK lysine residue(s) to Pm contributes ~14%. Although LBS interactions are important for efficient docking of SK, it appears that non-LBS interactions are the major source of the binding energy both for Pg and for Pm encounter. Previous equilibrium binding studies with α-domain-truncated SK showed that the LBS-independent interactions with the Pg/Pm protease domain largely reside in the SK α-domain, whereas the β- and γ-domains participate in LBS-dependent interactions with Pg/Pm kringles (71). It is likely that Pm-binding lysines other than the C-terminal Lys<sup>414</sup> reside in the SK β- and γ-domains.

SK-Pg and SK-Pm differ in their rate constants for the two conformational steps. The k<sub>2</sub> values for the SK-Pm complex were ~10 s<sup>-1</sup> for intact and Lys<sup>414</sup>-deleted SK and ~37 s<sup>-1</sup> in 6-AHA (59); the latter is comparable with k<sub>2</sub> for all the SK and SKΔK414 interactions with Pg in this study except those in benzamidine. For SK-Pm, the conformational restraint reflected by a low k<sub>2</sub> may be due to binding of a non-COOH-terminal SK lysine to K5, which also makes the SK-Pm encounter complex tighter. This restraint is absent in [Lys]Pg, suggesting no binding contribution from SK lysines other than Lys<sup>414</sup>. Benzamidine enhanced k<sub>2</sub> by 5- and 6-fold in SK binding to Pg and Pm, respectively (59). This enhancement was weaker with SKΔK414, suggesting that Lys<sup>414</sup> binding to K4 may release conformational restraints. The large k<sub>2</sub> value may contribute to a ~4-fold tighter K<sub>D, overall</sub> for SK-Pg in benzamidine compared with that for SK-Pg in 6-AHA and SKΔK414-Pg in all buffer systems. 6-AHA causes transition of [Glu]Pg from the compact α-form to the fully extended γ-form and of [Lys]Pg from the partially extended β- to the γ-form, whereas benzamidine keeps [Lys]Pg in the β-form (43). This suggests that the release of constraints on k<sub>2</sub> does not depend on α → β → γ conformational changes in Pg. The k<sub>2</sub>, k<sub>3</sub>, and k<sub>1</sub> values were similar in all data sets, indicating that these steps are LBS-independent.

In summary, we demonstrate here for the first time that the three-step kinetic model for the pathway of Pg binding to SK is substantially different from that of Pm binding with the main differences being a weaker encounter complex and increased off-rates for the conformational steps. Whereas cooperative Lys<sup>414</sup> and other lysine interactions with K4 and K5 and non-LBS interactions with the protease domain contribute to formation of the SK-Pm complex, the SK-Pg<sub>*</sub> complex assembly appears to be driven by non-LBS interactions and by SK Lys<sup>414</sup> binding to Pg kringle K4. Consistent with the experimentally determined K<sub>μ</sub> of ~2 μM for substrate Pg binding to the SK-Pg<sub>*</sub> complex and of 270 nM for Pg binding to the SK-Pm complex (7) and in the absence of a crystal structure of the SK-Pg<sub>*</sub> complex, we hypothesize that the weaker interaction with Pg in the catalytic complex results in expression of a pro-exosite that binds substrate Pg with lower affinity than the corresponding exosite on the SK-Pm complex.

Differences in affinity of the SK-Pg<sub>*</sub> and SK-Pm complexes may be important in their partitioning on bacterial surface proteins and in binding to host fibrinogen (ogen). Group A streptococcal M-like surface proteins bind Pg with high affinity, and the M1 subset lacking Pm-binding motifs binds fibrinogen (Fbg). This allows indirect activation by way of SK-Pg<sub>*</sub>-Fbg ternary complex formation (7, 77), which proceeds by Fbg binding to the SK-Pg<sub>*</sub> complex rather than SK recruitment on the Pg-Fbg complex (7). Group A Streptococcus SK exhibits significant polymorphism (78–80), and considerable differences exist among SK allelic variants in their efficiency of activating Pg and their recruitment in complexes with Fbg, Fbg fragment D, fibrin, and the plasminogen-binding group A streptococcal M protein (81). [Glu]Pg binds fibrin(ogen) through a K1 interaction, whereas K1 and K4 of [Lys]Pg and Pm are involved in fibrin(ogen) binding (74), and these differences are expected to influence localization of SK-Pg<sub>*</sub> and SK-Pm complexes. Future stopped-flow studies will identify how fibrin(ogen) and streptococcal surface proteins affect the pathways of SK-Pg<sub>*</sub> and SK-Pm formation and will be instrumental in characterizing these pathways in complexes with allelic SK variants.

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