Full Time Course Kinetics of the Streptokinase-Plasminogen Activation Pathway*

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Our previously hypothesized mechanism for the pathway of plasminogen (Pg) activation by streptokinase (SK) was tested by the use of full time course kinetics. Three discontinuous chromogenic substrate initial rate assays were developed with different quenching conditions that enabled quantitation of the time courses of Pg depletion, plasmin (Pm) formation, transient for-}

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ormation of the conformationally activated SK-Pg* catalytic complex intermediate, formation of the SK-Pm catalytic complex, and the free concentrations of Pg, Pm, and SK. Analysis of full time courses of Pg activation by five concentrations of SK along with activity-based titrations of SK-Pg* and SK-Pm formation yielded rate and dissociation constants within 2-fold of those determined previously by continuous measurement of parabolic chromogenic substrate hydrolysis and fluorescence-based equilibrium binding. The results obtained with orthogonal assays provide independent support for a mechanism in which the con-}

formationally activated SK-Pg* complex catalyzes an initial cycle of Pg proteolytic conversion to Pm that acts as a trigger. Higher affinity binding of the formed Pm to SK outcompetes Pg binding, terminating the trigger cycle and initiating the bullet catalytic cycle by the SK-Pm complex that converts the residual Pg into Pm. The new assays can be adapted to quantify SK-Pg activation in the context of SK- or Pg-directed inhibitors, effectors, and SK allelic variants. To support this, we show for the first time with an assay specific for SK-Pg* that fibrinogen forms a ternary SK-Pg*-fibrinogen complex, which assembles with 200-fold enhanced SK-Pg* affinity, signaled by a perturbation of the SK-Pg* active site.

Streptokinase (SK)² from Streptococcus pyogenes is a human host-specific, potent virulence factor in group A streptococcal infections, estimated to account for 500,000 deaths/year globally (1). SK from Streptococcus equisimilis used in the present studies is a group C streptococcal protein responsible for the potentially fatal infection in horses called strangles (2). S. equi-}

similis SK is most homologous (~90%) to phylogenetic cluster 1 SKs from S. pyogenes (3, 4). SK subverts the fibrinolytic system by specific binding to the catalytic domain of plasminogen (Pg) and by induction of non-proteolytic activation of the Pg zymo-}

gen by the molecular sexuality mechanism (5–9). SK inserts its NH₂-terminal Ile¹-Ala² into the NH₂-terminal binding cleft of the Pg catalytic domain such that Ile¹ forms a salt bridge with Pg Asp¹₉⁴ (chymotrypsinogen numbering) that induces confor-}

mational expression of the substrate binding site and the oxy-}

anion hole required for proteolytic activity (5–9). Although there is no crystallographic proof of this mechanism for SK, solution studies provide ample evidence for it, and for the con-}

formational activation of prothrombin by the Staphylococcus aureus activator, staphylocoagulase, crystal structure evidence is available (10).

Conformational activation of Pg in formation of the SK-Pg* complex initiates the ultimate conversion of Pg into the proteolytically activated product, plasmin (Pm) by a unique mecha-}

nism. The activated SK-Pg* complex binds another Pg molecule and cleaves it to Pm. Pm binds SK with very high affinity (Kp = 11–19 pm (11–13)), and formation of the SK-Pm complex is accompanied by expression of an exosite that assists in binding of Pg as a substrate of the SK-Pm complex, enabling proteolytic Pg activation into Pm, whereas Pm itself does not activate Pg (11, 14, 15). There are two modes of SK-Pg/Pm binding, one in the catalytic mode in SK-Pg* and SK-Pm complexes and the second in the substrate mode in the SK-Pg*-Pg and SK-Pm-Pg product-forming ternary complexes. The crystal structure of SK bound to the catalytic domain of Pm (µPm (5)) shows that SK interacts with µPm through three independently folded β-grasp domains, α, β, and γ, that are connected through two flexible linking sequences. In solution, SK has no stable domain structure (16), but when bound to the Pm catalytic domain, it organizes into a structure resembling a three-sided crater sur-}

rounding the Pm active site at the bottom (5).

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2 The abbreviations used are: SK, streptokinase; Pg, plasminogen; [Glu]Pg, intact native plasminogen; [Lys]Pg, native Pg lacking the NH₂-terminal 77 residues; Pm or [Lys]Pm, plasmin; Fbg, fibrinogen; FFR-CH₂Cl, d-Phe-Phe-Arg-CH₂Cl; FPR-CH₂Cl, D-Phe-Pro-Arg-CH₂Cl; 6-AHA, 6-aminohexanoic acid; Pg*, non-proteolytically activated form of the plasminogen zymogen; L85, lysine-binding site(s); pNA, para-nitroaniline; VLK-pNA, d-Val-Leu-Lys-pNA; FFR-Pm, Pm active site-blocked with d-Phe-Phe-Arg-CH₂Cl.
Binding of Pg/Pm to SK in both the catalytic and substrate modes is enhanced by interactions with lysine-binding sites (LBS) of primarily the 1, 4, and 5 kringle domains of Pg/Pm that are inhibited by the lysine analog, 6-aminohexanoic acid (6-AHA) (11–13, 17, 18). Formation of the SK-Pg* and SK-Pm catalytic complexes is enhanced 12–14-fold in affinity by the COOH-terminal Lys414 residue of SK binding to a Pg/Pm kringle that has not been definitively identified (13), whereas recent studies suggest that it may be kringle 4 (19). Binding of Pg in the substrate mode is mediated by interaction of Arg253, Lys256, and Lys257 of the SK 250-loop in the β-domain with kringle 5 of the substrate Pg (20–22). The dual modes of Pg as catalyst and substrate result in unique steady-state kinetics as a function of SK concentration (17, 18). At low concentrations of SK, Pg binds preferentially in the catalytic mode to form SK-Pg*, which proteolytically activates free Pg to Pm at a rate that increases to a maximum as the SK concentration is increased. At higher SK concentrations, the rate of Pm formation decreases, approaching zero, reflecting depletion of free Pg to function as the substrate (17, 18).

Continuous assays in the presence of a chromogenic substrate developed for steady-state kinetics of the SK-Pg activation mechanism allowed resolution of conformational formation of SK-Pg* from proteolytic Pm generation. Development of active site-fluorescently labeled Pg analogs enabled quantitation of the binding of SK to [Glu]Pg, [Lys]Pg, and Pm in the absence of proteolysis (11–13, 17, 18). These approaches demonstrated that (a) the affinity of SK for native [Glu]Pg is comparatively weak (KD ~130 nM) and LBS-independent, (b) [Lys]Pg binds SK more tightly (KD ~10 nM) and is partially LBS-dependent, and (c) Pm binds with very high affinity (KD = 11–19 pM) and is partially LBS-dependent. On this basis, a unified mechanism for the SK-Pg activation pathway was postulated (Fig. 1) (18). Rapid and reversible formation of the SK-[Lys]Pg* complex is the initiating event, followed by binding of substrate [Lys]Pg and its proteolytic activation to [Lys]Pm in the first catalytic cycle (Fig. 1, trigger). Because Pm binds SK in the catalytic mode with 500–900-fold higher affinity than [Lys]Pg, it displaces SK from SK-Pg*, forming SK-Pm, which initiates the second catalytic cycle (Fig. 1, bullet). Depending on the SK and Pg concentrations, only a few turns of the triggering cycle are required to deplete SK, which shuts off the triggering cycle, and the tightly bound SK-Pm complex activates the residual Pg to Pm.

The goal of the present work was to evaluate this mechanism for the SK-[Lys]Pg activation pathway by full time course kinetics under conditions where the distribution of reaction intermediates and products is dictated by the SK concentration. New chromogenic substrate assays that are orthogonal to those used previously were developed that distinguish between the reactants, intermediates, and products, using three different quenching conditions designed on the basis of the previously determined properties of the reaction species (11, 12, 17, 18). The results of quantitative characterization of the activation species over a 4–50 nM range of SK concentration exhibited behavior that supported the mechanism (Fig. 1). We propose that the structural organization of SK during Pg binding triggers conformational Pg activation and expression of the Pg substrate binding site and serves to order the reactions of the initial catalytic cycle of Pg activation catalyzed by SK-Pg*. The subsequent differentially higher affinity of SK for Pm compared with Pg binding in the catalytic mode directs the formation of the second SK-Pm catalytic complex that propagates complete activation of free Pg to Pm. In addition, we show that the assays developed can be applied to investigate the mechanism through which fibrinogen regulates [Lys]Pg activation by SK.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Characterization**

Native SK was purified from outdated therapeutic SK (S. equisimilis strain H46A; Diapharma) by affinity chromatography on SulfoLink gel (Pierce) to which Pm was linked though its active site by Nα-((acetylthio)acetyl)-D-Phe-Phe-Arg-CH₂Cl as described (11, 12, 23). Human [Glu]Pg carbohydrate form 2 was purified from normal plasma by published procedures (12, 17, 23–27). Activation of 10 μM [Glu]Pg to [Lys]Pm with 90
Assays for Full Time Course Kinetics

Three assays were developed to quantitate SK-[Lys]Pg reaction species: SK\textsubscript{Pm\_Free}, SK\textsubscript{Pm\_Pre} and SK\textsubscript{Pm\_Total} (Pm + SK-Pm). Assays were performed by incubating [Lys]Pg and SK for various times and quenching the reaction by the addition of 200 nM FFR-Pm and 10 mM 6-AHA for 2 min to dissociate the SK-Pg* complex and to inhibit additional Pm formation, respectively. Control assays containing known concentrations of Pm or SK-Pm in the presence of 10 mM 6-AHA were inhibited 20% ± 2% compared with assays without 6-AHA. The rates for this assay were corrected for the 6-AHA effect, which is most likely due to weak inhibition of the Pm catalytic site in a non-competitive (39) or, more recently, a competitive mechanism (40). FFR-Pm had no effect on the rates for Pm or SK-Pm. Prequenched reactions in which [Lys]Pg was preincubated with 200 nM FFR-Pm, 10 mM 6-AHA, and VLK-pNA and the assay initiated with SK resulted in Pm\textsubscript{Total} rates representing <0.5% of the Pm\textsubscript{Total} maximum rate. Such prequenching was used to define the rates at time 0 in the full time course experiments. Pm\textsubscript{Total} concentrations were calculated using a rearrangement of the Michaelis-Menten equation,

\[
[E]_o = \frac{V_{	ext{cat}}(K_m + [S]_o)}{k_{	ext{cat}}[S]_o}
\]

where \([E]_o\) is the total Pm formed at each quench time, \([S]_o\) is the VLK-pNA concentration, and \(V_{	ext{cat}}\) is the measured initial velocity. The previously published kinetic parameters \((K_m = 140 ± 10 \mu M\) and \(k_{	ext{cat}} = 16.9 ± 0.5 \text{ s}^{-1}\) for Pm were used (11). The kinetic parameters for SK-Pm previously determined are \(K_m = 300 ± 50 \mu M\) and \(k_{	ext{cat}} = 34 ± 2 \text{ s}^{-1}\) (11). At 50 \(\mu M\) VLK-pNA, the calculated initial rate for SK-Pm is 9.2% greater than that of free Pm, which was within the experimental error of the parameters. For all Pm preparations used in the current studies, the active Pm concentration was calculated from the initial rate using the kinetic constants above that were obtained with Pm preparations that were active site-titrated with nitrilotriphenyl-p'-guanidinobenzoate or fluorescein mono-p-guanidinobenzoate (11).

Assay 2, SK-Pm—SK-[Lys]Pg reactions were quenched with 200 nM FFR-Pm and 100 nM AP for 30 s to dissociate the SK-[Lys]Pg* complex and to inactivate free Pm, respectively. This is possible because SK-Pm is resistant to inactivation by AP (41). FFR-Pm and AP at the concentrations used had no effect on the rates of VLK-pNA hydrolysis by SK-Pm. Control assays showed that 20 nM Pm was 97% inactivated by 50 nM AP after 30 s under these conditions. Prequenched reactions in which [Lys]Pg was preincubated with 200 nM FFR-Pm, 100 nM AP, and VLK-pNA and the assay was initiated with SK resulted in SK-Pm rates of VLK-pNA hydrolysis <0.5% of the SK-Pm maximum activity.

For determination of SK-Pm and Pm\textsubscript{Pm\_Free}, additional results were necessary because of the high affinity of SK for Pm \((K_D = 11–19 \text{ pm}) (11–13)\). Titrations of 7.8 and 20 nM active Pm with SK were performed using the quenching conditions for Assay 2. The rates as a function of SK concentration were normalized to
the maximum rate at saturating SK concentration and fit simultaneously by the quadratic binding equation to obtain the fitted maximum rates and the stoichiometric factor, with $K_D$ fixed at 12 pm. Pm preparations typically show stoichiometric factors of 1.3 mol of SK/mol of Pm, despite active site titration and high purity by SDS-PAGE (11). In the present case, a value of 1.42 ± 0.03 mol/mol was obtained. This value was rather high for two reasons. First, assuming that the SK is fully active and that Pm was 81 ± 2% active accounts for a stoichiometric factor of 1.23 mol of SK/mol of active Pm. Second, fitting of the two titrations simultaneously with the quadratic binding equation used the active concentrations of Pm, which increased the stoichiometric factor from 1.23 to the value of 1.42 given above. The purpose of performing the analysis in this way was to obtain a single factor to correct the rates in Assay 2, because these rates only measure active species. The rates obtained in Assay 2 at sub-saturating SK concentrations were multiplied by 1.42 to adjust for the slope of the linear part of the titration with SK. $Pm_{Free}$ was calculated by subtracting the concentration of SK-Pm (Assay 2) from $Pm_{Total}$ (Assay 1).

Assay 3, SK-Pm + SK-Pg*—This assay is based on the protection of SK-Pg* and SK-Pm from inactivation by AP (41, 42). SK-[Lys]Pg reactions were quenched by the addition of 100 nM AP for 30 s to inactivate $Pm_{Free}$. Prequenched reactions in which [Lys]Pg was preincubated with 100 nM AP and VLK-pNA and the assay was initiated with an excess of SK resulted in rates representing the maximum concentration of SK-Pg* formed.

To obtain a different measurement of SK-Pg*, 7.8 or 20 nM [Lys]Pg, 100 nM AP, and 50 μM VLK-pNA were titrated with 0–90 nM SK. These prequenched reactions inactivated any $Pm_{Free}$ leaving SK-Pg* as the only active species. The continuously monitored initial rates of 50 μM VLK-pNA hydrolysis were linear, indicating no significant Pg activation, which would have produced parabolic progress curves (13, 17, 18, 20). The maximum rates at saturating levels of SK were normalized to the maximum Pg concentrations determined from the fit by the quadratic binding equation. The rates measured for Assay 2 (SK-Pm) were subtracted from those obtained in Assay 3 (SK-Pm + SK-Pg*) to obtain the rate for SK-Pg*. SK-Pg* concentrations were calculated using new kinetic parameters for VLK-pNA extended to 10 mM substrate (4.3 × $K_m$), $K_m = 2.32 ± 0.14$ mm and $k_{cat} = 40.8 ± 0.8$ s⁻¹.

$SK_{Free}$ and $Pg_{Free}$

$SK_{Free}$ and $Pg_{Free}$ concentrations were obtained from the conservation equations, [SK$_{Free}$] = [SK]$_o$ - [SK-Pm] - [SK-Pg*] and [Pg$_{Free}$] = [Pg]$_{o}$ - [Pm]$_{Total}$ - [SK-Pg*], where [SK]$_o$ and [Pg]$_o$ are the time 0 concentrations.

Additional Corrections

For progress curves that converted all of the Pg to Pm, the averaged maximum rates from Assay 2 (SK-Pm) near the end of the reactions were normalized to the maximum rates from Assay 3 (SK-Pm + SK-Pg*). This assumed that at the plateau of SK-Pm formation near the end of the full activation reactions, the concentration of the transiently formed SK-Pg* complex was completely depleted, such that the rates from Assays 2 and 3 would be equivalent, as shown by the results. The maximum correction was ≤7% for all of the full-activation experiments.

**Fitting of the Mechanism for the SK-[Lys]Pg Activation Pathway**

Five time course data sets at different SK concentrations, titrations of SK-Pg* formation at 7.8 and 20 nM Pg, and an SK titration of 1 nM SK-Pm formation in the presence of 10 mM 6-AHA were fit simultaneously by numerical integration and least squares fitting of the mechanism below with KinTek Explorer version 3.0 (43, 44).

\[
SK + Pg \xrightarrow{k_1} SK \cdot Pg^* \quad \text{(Eq. 2)}
\]

\[
SK \cdot Pg^* + Pg \xrightarrow{k_2} SK \cdot Pg^* \cdot Pg \quad \text{(Eq. 3)}
\]

\[
SK \cdot Pg^* \cdot Pg \xrightarrow{k_3} SK \cdot Pg^* + Pm \quad \text{(Eq. 4)}
\]

\[
SK + Pm \xrightarrow{k_4} SK \cdot Pm \quad \text{(Eq. 5)}
\]

\[
SK \cdot Pm + Pm \xrightarrow{k_5} SK \cdot Pm \cdot Pm \quad \text{(Eq. 6)}
\]

\[
SK \cdot Pm \cdot Pm \xrightarrow{k_6} SK \cdot Pm + Pm \quad \text{(Eq. 7)}
\]

Pg binding to SK in the catalytic mode to form the conformationally activated SK-Pg* complex is represented as a single, rapid equilibrium step with dissociation constant $K_D = k_{-1}/k_1$ (Equation 2). The second step is binding of Pg as the substrate of the SK-Pg* catalytic complex ($K_s = k_{-2}/k_2$; Equation 3) to generate Pm with catalytic rate constant $k_{Pm}$ (Equation 4). This reaction is bimolecular with a rate constant of $k_{Pg}/K_s$ because the Pg concentration of 20 nM used in the kinetic studies was apparently much lower than the $K_s$ as shown by the inability of the mechanism to fit this parameter. Pm generated in Equation 4 binds tightly to SK in the catalytic mode even in the presence of 10 mM 6-AHA (Equation 5). The SK-Pm catalytic complex then recognizes free Pg as its substrate ($K'_s = k_{-4}/k_4$; Equation 6) and converts it proteolytically into Pm (Equation 7), which is also a bimolecular reaction with rate constant $k_{Pm}/K'_s$, because the [Lys]Pg concentration used was much lower than the value of $K'_s$ of 270 nM determined previously (18).

**Fitting Strategy**

Initial constraints for fitting were made on the basis of the two cycles catalyzed first by SK-Pg* (Equations 2–4) and second by SK-Pm (Equations 5–7). The on- and off-rate constants for SK binding to Pg, $k_1$ and $k_{-1}$ (Equation 2), were set to vary in a fixed ratio (a feature of the software), as were Pg substrate bind-
ing $k_2$ and $k_{-2}$, and the catalytic rate constant $k_{pg}$ with all three varied in a different fixed ratio. The analogous SK-Pm $k_3$ and $k_{-3}$, the Pg substrate binding $k_4$ and $k_{-4}$, and $k_{Pm}$ were set to vary in a separate fixed ratio. All separate binding steps were initially assigned 1 nm$^{-1}$ s$^{-1}$ on-rate constants. Initial parameters for the fit were those determined here. Because few values of rate or affinity are available for Pg substrate binding to SK-Pg* and SK-Pm, an off-rate constant to yield a $K_p$ value of 270 nm previously determined was used (18). Later in the analysis, an off-rate constant for Pg substrate binding to SK-Pg* was identified by the lowest dissociation constant (2 μM) that did not alter detectably the fit of the most sensitive progress curves, and the parameters for this step were fixed at this value. In the final analysis, the constants for Pg substrate binding to SK-Pm and the bimolecular rate constant ($k_{Pm}/K_p$) were constrained as a group to vary in a constant ratio. All other constants were fitted (see Table 1).

**SK-Pg*-Fbg Ternary Complex Experiments**

Prequenching of mixtures of 5 nm [Lys]Pg with 100 nm AP and 50 μM VLK-pNA in the absence and presence of fixed concentrations of Fbg was followed by preincubation for 2 min before initiation of the assay by increasing SK concentrations. For a titration of 1 nm [Lys] at a fixed SK concentration of 60 nm, mixtures of [Lys]Pg, Fbg, and VLK-pNA were prequenched with AP in the same way except that the Fbg concentration was varied. The results were analyzed by non-linear least squares fitting by an iterative model using SCIENTIST software. The equations for the dissociation constants $K_{A}$, $K_{P}$, and $K_{C}$, the two $K_{cat}/K_{m}$ values for the two active product-forming complexes (SK-Pg* and SK-Pg*-Fbg), and the mass balance equations were simultaneously solved by the software. $K_{D,calc}$ was calculated from detailed balance, $K_{D,calc} = K_{A} \times K_{P}/K_{C}$. $K_{C}$ was fixed at its determined value.3

**RESULTS**

**Assays for SK-[Lys]Pg Full Time Course Kinetics**—Chromogenic substrate (VLK-pNA) initial rate assays were developed to selectively measure the concentrations of plasmin, $Pm_{Total} = Pm_{Free} + SK-Pm$ (Assay 1), SK-Pm (Assay 2), and SK-Pm + SK-Pg* (Assay 3), based on the previously determined properties of these SK-[Lys]Pg activation intermediates and products (11, 12, 17, 18). Different combinations of 200 nm active site-blocked Pm, prepared with D-Phe-Phe-Arg-CH$_2$Cl (FPR-Pm), a 10 mm concentration of the Pg/Pm kriple-binding lysine analog 6-AHA, or 100 nm AP, the rapid and irreversible physiological serpin inhibitor of Pm, were used to quench the reactions.

For Assay 1, quenching with FPR-Pm and 6-AHA dissociated the transiently formed, conformationally activated SK-Pg* complex and inhibited Pm formation. This assay was based on a 10–20-fold weakening of the 10 nm $K_p$ of SK-Pg* formation, in which Pg is bound in the catalytic mode to SK, by 10 nm 6-AHA blocking the LBS of a Pg kringle. This prevents the affinity-enhancing interaction with the COOH-terminal Lys$^{414}$ residue of SK (13, 17, 18). FPR-Pm binds in the catalytic mode to SK with a $K_p$ value of 11–19 pm, 500–900-fold tighter than SK-Pg* (11–13, 17). Binding of SK to Pm is similarly weakened by 10 mM 6-AHA blocking the Lys$^{414}$-kringle interaction, but Pm still maintains an LBS-independent 0.2–0.3 nm $K_p$ value for SK (11–13). The net effect of quenching with 6-AHA and FPR-Pm is dissociation of SK-Pg* and sequestering of free SK in the SK-FPR-Pm inactive complex. One consequence, however, is that the use of 10 nm 6-AHA as a quenching reagent only in Assay 1 results in 10–20-fold weakening of SK binding to Pm. Although this might affect the distribution of PmFree and SK-Pm measured in Assay 1, it does not affect the concentration of PmTotal. Previous studies show that the off-rate constant for displacement of Pm by FPR-Pm is ~0.0008 s$^{-1}$, slow enough for the dissociation of Pm to be negligible in the 2-min quench time (45). In any case, only PmTotal appears in the conservation equations used to calculate $K_{Free, Pg^*}$ and PmFree. This might also affect the reaction pathway to a small extent.

Assay 2 measures the SK-Pm complex by quenching with 200 nm FPR-Pm and incubation with 100 nm AP for 30 s before initiating the assay by substrate addition. The SK-Pg* complex is dissociated by FPR-Pm due to the much higher affinity of SK for FPR-Pm compared with SK-Pg*, as described above. Pm (10 nm) was ≥99% inactivated by 100 nm AP, whereas SK-Pm is protected from inactivation by AP (41), leaving SK-Pm the only active species.

An additional calibration was required for determination of the concentrations of SK-Pm and PmFree. Titration of 7.8 and 20 nm active Pm with SK were performed with quenching as described for Assay 2 (Fig. 2A). The titrations were normalized to the maximum rates at saturating SK and fit by the quadratic binding equation with the stoichiometric factor and fitted maximum rate as parameters and the value of $K_p$ fixed at 12 pm (11). Stoichiometric factors for SK binding to Pm are often as high as 1.3 mol of SK/mol of Pm, although Pm preparations were pure by SDS-PAGE and active-site-titrations were performed (11). In the present case, a value of 1.42 ± 0.03 mol of SK/mol of active Pm was obtained (Fig. 2A). This value incorporates the 81 ± 2% activity of the Pm preparation in the stoichiometric factor and the use of the active Pm concentration in the 7.8 and 20 nm acceptor concentrations for fitting the titrations with SK. The analysis was done in this manner to obtain a single factor based on only the active concentrations of Pm and SK-Pm to correct the rates in Assay 2. The rates obtained with Assay 2 at subsaturating SK concentrations were multiplied by 1.42 to adjust the slope of the linear part of the titrations, and the concentration of PmFree was obtained by subtracting SK-Pm (Assay 2) from PmTotal (Assay 1). Titrations with SK using the same preparation of Pm were done in the presence of 10 mM 6-AHA and quenched with AP (Fig. 2B). Titration of 1 nm Pm with SK gave a $K_p$ value of 0.33 ± 0.14 nm, corresponding to a ~25-fold lower affinity than the mean value of 13 pm in the absence of 6-AHA (11–13).

Assay 3 measures the sum of SK-Pm and SK-Pg* concentrations by quenching with 100 nm AP, because both complexes are protected from inactivation by AP, whereas PmFree is inactivated (41, 42). Titrations of prequenched mixtures of 7.8 or 20 nm [Lys]Pg with 100 nm AP and 50 μM VLK-pNA were initiated by the addition of increasing concentrations of SK (Fig. 2C). The titrations were fit simultaneously by the quadratic binding

3 J. I. Creamer, S. D. Bouldin, A. A. Maddur, and P. E. Bock, unpublished results.
equation to the data normalized to the total Pg concentration obtained from the fit because it was not possible to determine the maximum independently from the data as done for SK-Pm. Fitting of the normalized data in this way did not change the $K_D$ value or the relative error in the fitted parameters, which were SK-Pg* maximum concentrations of 7.8 ± 0.7 nM, 20 ± 1 nM, and a $K_D$ value of 9 ± 2 nM (Fig. 2C). The $K_D$ value was indistinguishable from $K_D$ values of 9–10 nM determined previously by competitive equilibrium binding of native and fluorescently labeled [Lys]Pg (13, 17). The concentration of SK-Pg* was determined from the rate obtained in Assay 3 using new kinetic parameters determined for VLK-pNA. The results supported the capacity of Assay 3 to quantitate SK-Pg*, albeit with significant experimental error due to its 6.3-fold lower chromogenic substrate activity ($k_{cat}/K_m$) compared with SK-Pm.

**Assay Stability and Reproducibility**—The stability and reproducibility of the assays were investigated by incubation of 10 nM Pm or SK-Pm with the quenching components as a function of time (Fig. 3, A and B). Pm and SK-Pm were each incubated with FFR-Pm, 6-AHA, AP, or 6-AHA and a combination of AP, FFR-Pm, and 6-AHA for up to 60 min before the chromogenic substrate assay. The concentrations of quenching components were 100 nM FFR-Pm, 10 mM 6-AHA, 50 nM AP, and 6-AHA and a combination of AP, FFR-Pm, and 6-AHA for 30 s before adding 10 mM 6-AHA (black squares). The assays containing AP had no detectable activity, and the symbols were superimposable. Assays were initiated by the addition of 50 μM VLK-pNA, those containing 10 mM 6-AHA were corrected for 20 ± 2% inhibition, and all results are shown as fractional activity relative to the initial rate at time 0. B, SK-Pm was prepared by mixing 200 nM Pm with 470 nM SK and incubated at 10 nM as described in A in the absence and presence of the same quenching components shown by the same symbols in A. Assays were performed and analyzed as described under "Experimental Procedures."

**Streptokinase-Plasminogen Activation Pathway**

![Streptokinase-Plasminogen Activation Pathway](image)
Streptokinase-Plasminogen Activation Pathway

Time course experiment at 11.7 nM SK and 20 nM [Lys]Pg is approaching zero as the concentration of [Lys]PgFree to act as "Experimental Procedures." (the black lines. B FIGURE 4. Deconvolution of a representative full time course experiment. A, results of the full time course of all six reaction species as a function of time for activation of 20 nM [Lys]Pg by 11.7 nM SK with the global fit indicated by the black lines. B, the time course of the concentration of SKFree ([SKFree]) as shown as purple circles. C, the time course of the concentration of PmFree ([PgFree]) is shown as dark blue circles. D, the time course of the concentration of PmFree ([PgFree]) is shown as orange triangles. E, the time course of the concentration of SK-Pg* ([SK-Pg*]) is shown as green triangles. F, the time course of the concentration of SK-Pm ([SK-Pm]) is shown as red squares. G, the time course of the concentration of PmTotal ([PmTotal]) is shown as turquoise squares. Assays and global analysis were performed as described under "Experimental Procedures."

sioning from substoichiometric to excess SK concentration over that of [Lys]Pg. Because [Lys]Pg acts as both catalyst and substrate, at [SK] = 0.5 × [[Lys]Pg], the rate of Pm formation catalyzed by SK-[Lys]Pg* increases to a maximum at [SK] near 0.5 × [[Lys]Pg], and then decreases at [SK] ≫ [[Lys]Pg], approaching zero as the concentration of [Lys]PgFree to act as substrate is depleted (18).

Five full time course experiments were fit globally by combined numerical integration and least squares fitting of the mechanism (Equations 2–7) (43, 44). Additional data included the SK-Pm activity titration in the presence of 6-AHA (Fig. 2B) and two SK-Pg* activity titrations (Fig. 2C). A representative full time course experiment at 11.7 nM SK and 20 nM [Lys]Pg is shown in Fig. 4A along with the global fit for the concentrations of six deconvoluted activation species: SKFree, PmFree, SK-Pg*, SK-Pm, and PmTotal (Fig. 4, B–G). At time 0, the measured values of the concentrations of SKFree, PmFree, SK-Pg*, and SK-Pm were 5.0, 13.3, and 6.6 nM, respectively, from which a Kd value of 10 nM was calculated, in reasonable agreement with the Kd (6 nM) from the global analysis. There were obvious lags in the time courses of PmFree, PmFree, SK-Pm, and PmTotal which were accounted for by the sequential generation of Pm for all of the Pm species and the faster release of PmFree from total depletion of SKFree and SK-Pg*. As can be seen from these reactions at saturating SK concentration with respect to that of Pg, the activation process catalyzed by SK-Pg* ceases when this complex is fully depleted at ~300 s and one SK equivalent of SK-Pm has been formed, which remains constant, whereas PmFree and PmTotal approach completion. The concentration of PmTotal at the end of the reactions (19.6 nM; mean of 5 points at 600–1080 s) was distributed as 43% PmFree, 57% SK-Pm, where the latter value corresponded to the initial 58% ratio of [SK] to [Pg]. The calculated Kd value for SK binding to Pm at the end of the reactions was 310 pm, in good agreement with the global fit value of 290 pm. This substantiated the assumption that minimal dissociation of SK-Pm by FFR-Pm occurred in the 2-min quenching time for Assay 1 because the off-rate constant is ~0.0008 s⁻¹ (45).

The global fit of the five full time course and three titration data sets (715 assays) is shown in Fig. 5, A–H, with the parameters listed in Table 1. The results show how the pathway of Pm formation is initially accelerated at substoichiometric SK concentrations (Fig. 5, A and B) and subsequently inhibited at higher SK concentration (Fig. 5, D and E). At saturating SK, one equivalent of SK-Pm is formed and is stable after that, and the activation reaction continues until all of the Pm is converted to PmFree and SK-Pm (Fig. 5, A and B). At equimolar (20 nM) SK and Pg concentrations, generation of SK-Pm and PmTotal coalesces, and this is maintained at higher SK concentrations, although the rate of PmTotal formation decreases (Fig. 5, C–E). Over the high SK concentration range, SK-Pg* increases at time 0 until all Pg is bound in SK-Pg*, and PmFree is reduced to an undetectably low concentration (Fig. 5E). SK-Pm continues to be formed, whereas the concentration of PmFree is undetectably low, until all of the remaining SK-Pg* is converted into SK-Pm (Fig. 5E).

Novel Applications of the Assays; Quantitation of the Transition from the SK-Pg* to the SK-Pm Catalytic Complex during the [Lys]Pg Full Time Course—The assays developed are capable of determining all of the SK-[Lys]Pg substrate depletion, transient intermediates, and products of the activation pathway. They can also accommodate a variety of different experimental designs by modification and recalibration of the assays. For example, the transition of the SK-Pg* to the SK-Pm catalytic complex during the time course of [Lys]Pg activation can be measured using Assay 3 (Fig. 6). This is distinct from the use of Assay 3 in prequenched reactions from which SK-Pg* was measured and Pm formation was blocked. Quenching with only AP inactivated the generated PmFree and yielded the sum of the catalytic complexes weighted by their contribution to VLK-pNA hydrolysis. The resulting curve at 5 nM [Lys]Pg and 10 nM SK exhibited a small lag as SK-Pg* was depleted, approaching a final plateau as SK-Pm became the sole catalyst (Fig. 6A). At higher SK concentration (40 nM), the rate of transition was slower and reached the same end point. The addition of the two
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TABLE 1
Summary of kinetic and binding constants from global fitting of SK-[Lys]Pg full time courses and activity titrations for SK-Pg* and SK-Pm

| Reaction step | Parameters | Global fit values | Constraints |
|---------------|-----------|-------------------|-------------|
| SK + Pg ⇌ SK-Pg* (Eq. 2) | $k_1$ | $0.10 \pm 0.02 \text{ nM}^{-1} \text{s}^{-1}$ | 
| | $k_{-1}$ | $0.60 \pm 0.17 \text{ s}^{-1}$ | 
| | $K_D$ | $6 \pm 2 \text{ nM}$ | 
| SK-Pg + Pg ⇌ SK-Pg·Pg (Eq. 3) | $k_2$ | $1 \text{ nM}^{-1} \text{s}^{-1}$ | F
| | $K_S$ | $2000 \text{ s}^{-1}$ | F
| SK-Pg·Pg → SK-Pg + Pm (Eq. 4) | $k_3$ | $1.17 \pm 0.17 \text{ M}^{-1} \text{s}^{-1}$ | 
| SK + Pm ⇌ SK·Pm (Eq. 5) | $k_1$ | $0.34 \pm 0.27 \text{ s}^{-1}$ | 
| | $K_D$ | $290 \pm 280 \mu\text{M}$ | 
| SK·Pm + Pg ⇌ SK·Pm·Pg (Eq. 6) | $k_4$ | $1.00 \pm 0.09 \text{ M}^{-1} \text{s}^{-1}$ | C1
| SK·Pm·Pg → SK·Pm + Pm (Eq. 7) | $k_5$ | $270 \pm 25 \text{ s}^{-1}$ | C1
| | $K_S$ | $270 \pm 35 \text{ nM}$ | 
| | $k_{	ext{cat}}/k_{	ext{m}}$ | $2.4 \pm 0.4 \text{ M}^{-1} \text{s}^{-1}$ | C1

Table 1: Summary of kinetic and binding constants from global fitting of SK-[Lys]Pg full time courses and activity titrations for SK-Pg* and SK-Pm. Listed are the individual reaction steps and corresponding equations under “Experimental Procedures.” Parameters are listed with their global fit values. Error in the parameters was propagated and represents the 2×S.E. F represents parameters that were fixed at the listed values. Constraint C1 is indicated for parameters that were grouped and varied in a fixed ratio. Fitting was performed as described under “Experimental Procedures.”

TABLE 1 Summary of kinetic and binding constants from global fitting of SK-[Lys]Pg full time courses and activity titrations for SK-Pg* and SK-Pm. Listed are the individual reaction steps and corresponding equations under “Experimental Procedures.” Parameters are listed with their global fit values. Error in the parameters was propagated and represents the 2×S.E. F represents parameters that were fixed at the listed values. Constraint C1 is indicated for parameters that were grouped and varied in a fixed ratio. Fitting was performed as described under “Experimental Procedures.”

curves in Fig. 6 to the experimental data set required minor changes in the output equations to account for the amplitude of the biphasic reactions, which arose from the disappearance of SK-Pg* and appearance of SK-Pm. Two amplitude factors were added to give $f_1 \times [\text{SK-Pg}^*] + f_2 \times [\text{SK-Pm}]$. Amendment of the data set and the mechanism by the addition of the amplitude factors had no effect on the fitted parameters or experimental error compared with those obtained from the global analysis. The fitted amplitude factors, with all other parameters in Table 1 fixed, correspond to the rates of VLK-PnA hydrolysis by SK-Pg* ($f_1$) and SK-Pm ($f_2$), which were 0.828 and 4.13 nM s$^{-1}$, respectively. The ratio of the rates was $5 \pm 6$ ($2\times$S.E.), with the large error due to that for the SK-Pg* complex. The ratio was indistinguishable from the value of 5.6 calculated from the amplitudes, chromogenic substrate concentration, and the kinetic parameters for SK-Pg* and SK-Pm using the Michaelis-Menten equation.

Simulation of the mechanism over a 100-fold range of SK concentration starting at equimolar [Lys]Pg (5 nM) showed relatively rapid transition of SK-Pg* to SK-Pm with a reduced final maximum amplitude due to ~20% of the Pn being converted into Pm$_{\text{free}}$, that was inactivated by AP quenching and the remaining ~80% SK-Pm. The transition was slower at 50 nM SK and exceedingly slow at 500 nM, where the rates representing SK-Pg* depletion and SK-Pm formation were linear. This behavior is explained by the sequestering of P$_{\text{Bfree}}$ in SK-Pg* by excess SK. This sequestering inhibits both catalytic cycles by depleting P$_{\text{Bfree}}$ needed to bind to the catalytic complexes in the substrate mode.

Mechanism of the Effect of Fibrinogen on SK-[Lys]Pg Activation—The SK-Pg activation mechanism is regulated by fibrinogen and fibrin through a SK-Pg*-fibrin(ogen) ternary complex (46–50). To demonstrate further the utility of the assays developed, Assay 3 was used in the prequenching design with AP to examine binding of Fbg to the SK-Pg* complex. The results show titrations with SK of 5 nM [Lys]Pg measured by the initial velocity of 50 μM VLK-PnA hydrolysis by SK-Pg* in the absence and presence of four fixed concentrations of Fbg (Fig. 7A) and a titration with Fbg of 1 nM [Lys]Pg and SK fixed at 60 nM (Fig. 7B). The titrations with SK produced a family of hyperbolic curves with increasing amplitudes up to 1–2 μM Fbg (Fig. 7A), whereas the Fbg titration showed an apparently tighter interaction. The results (Fig. 7, A and B) were simultaneously fitted by a minimal random addition ternary complex mechanism (Fig. 7C). $K_A$, $K_{Fbg}$, and the bimolecular rate constants ($k_{cat}/k_{m}$) for the active SK-Pg* binary complex and the SK-Pg*-Fbg ternary complex for VLK-PnA hydrolysis were fit-
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**FIGURE 6. Transition of the SK-Pg* catalytic cycle to the SK-Pm catalytic cycle during [Lys]Pg activation.** A, time course of 5 nM [Lys]Pg activation by 9.9 nM SK quenched with 100 (●) or 190 nM AP (○) for 30 s before the addition of 50 μM VLK-pNA. Activity is expressed as the observed initial rate of chromogenic substrate hydrolysis (v_{obs}), with the time 0 value obtained by prequenching. The solid black line was calculated from the global fit parameters in Table 1 with the mechanism including the results in this figure. The dashed black lines show the calculated curves for SK-Pg* depletion and SK-Pm formation. Results are also shown for activation of 5 nM [Lys]Pg by 40 nM SK prequenched and quenched with 100 (filled red triangles) or 190 nM AP (open red triangles), and the calculated fit is shown by the solid red line. Calculated curves for SK-Pg* depletion and SK-Pm formation are shown by the dashed red lines. B, simulated time courses of activation reactions at 5 nM [Lys]Pg and 5 (black lines), 50 (red lines), or 500 nM SK (blue lines). Calculated curves for SK-Pg* depletion and SK-Pm formation are indicated by the dashed lines in the corresponding colors. The parameters were those in A. Assays were performed as described under “Experimental Procedures.”

- **FIGURE 7. Random addition ternary complex mechanism for interactions of SK, [Lys]Pg, and Fbg.** A, initial rates (v_{obs}) of 50 μM VLK-pNA hydrolysis were measured at fixed concentrations of Fbg (protomer) at 0 (●), 50 (○), 200 (△), 1000 (□), and 2000 nM (■) in assay buffer as a function of total concentration of SK ([SK]_{tota}). The assays were performed by prequenching Pg with 100 nM AP and VLK-pNA for 2 min before initiating the reaction with increasing concentrations of SK, B, activity titration, as in A, for 1 nM Pg and 60 nM SK as a function of total Fbg (protomer) concentration ([Fbg]_{tota}) in assay buffer. The solid lines represent the simultaneous fit of data in A and B, C, a ternary complex mechanism for interactions of SK, [Lys]Pg, and Fbg. The value of K_{C}, for [Lys]Pg binding to Fbg was fixed at 19,000 nM based on unpublished results. Fitted parameters K_{A} and K_{B} (nM) and the bimolecular rate constants for VLK-pNA substrate hydrolysis (k_{cat}/K_{m} in μM^{-1} s^{-1}) are shown for SK-Pg* and SK-Pg*Fbg. VLK-pNA substrate is shown as S in the green oval, and the product (pNA) is the P in the orange oval. K_{C} (nM) was calculated using detailed balance. Assays were performed and analyzed as described under “Experimental Procedures.”

Although it has been known for a long time that an SK-Pg*Fbg ternary complex is formed, this conclusion was inferred from descriptive studies, including native gel electrophoresis (50), kinetic studies that were not quantitated (49), or qualitative studies using bacteria (48, 51). A few kinetic studies have been done that showed Fbg stimulation of [Lys]Pg activation by SK of 2- or 5.6-fold, which were attributed to other aspects of the SK-Pg mechanism or to entirely different mechanisms (46, 47). It should be noted that we eliminated various sequential mechanisms that included formation of a ternary SK-Pg*Fbg complex, all of which failed to fit the results. None have addressed the mechanism presented here quantitatively, which establishes for the first time the random addition ternary complex mechanism that greatly facilitates assembly of the...
TABLE 2
Parameters for the mechanism of assembly of the ternary complex among SK, [Lys]Pg, and Fbg

Listed are the fitted dissociation constants for the ternary complex mechanism (Fig. 7C) (designated T under “Source”) \( K_a \) and \( K_c \) were fitted, the dissociation constant \( K_c \) was fixed (F under “Constraint”) at its determined value, and \( K_a \), calc. was calculated from detailed balance. The fitted bimolecular rate constants for VLK-pNaseA plasminogen (Pm) and plasminogen (Pg) trypsin complexes by SK (20). The SK-Pg-Fbg complex at saturating SK (500 nM) and Fbg (2 μM) are also listed (data not shown). Error in the parameters was propagated and represents \( 2 \times \) S.D. Experiments were performed and analyzed as described under “Experimental Procedures” and “Results.”

| Parameter | Fitted value | Source | Constraint |
|-----------|--------------|--------|------------|
| \( K_a \) | 24 ± 8 nM | T | |
| \( K_c \) | 94 ± 25 nM | T | |
| \( K_c \), calc. | 19 ± 6.3 μM | T | F |
| \( K_a \)(sk-Pg') | 0.12 ± 0.06 nM | T | |
| \( k_{cat}/K_m \)(sk-Pg') | 0.018 ± 0.001 μM\(^{-1}\) s\(^{-1}\) | T | |
| \( k_{cat}/K_m \)(sk-Pg'-Fbg) | 0.055 ± 0.002 μM\(^{-1}\) s\(^{-1}\) | T | |
| \( K_{v_{obs} versus S_o}} \)(sk-Pg') | 2.32 ± 0.14 μM | \( v_{obs versus S_o}} \)(sk-Pg') | 1.0 ± 0.1 μM |
| \( K_{v_{obs} versus S_o}} \)(sk-Pg'-Fbg) | 40.8 ± 0.8 s\(^{-1}\) | \( v_{obs versus S_o}} \)(sk-Pg'-Fbg) | 56 ± 1 s\(^{-1}\) |

SK-Pg'-Fbg complex and has broad significance in streptococcal pathogenesis.

DISCUSSION

The results obtained by new assays that are orthogonal to those used previously provide independent support for the validity of the mechanism of the SK-initiated [Lys]Pg activation pathway previously proposed (17, 18). The previous studies were based on continuous steady-state kinetics in the presence of a chromogenic substrate and on competitive equilibrium binding experiments with the native proteins using active site-labeled Pg/Pm analogs as binding probes. Importantly, the kinetic and binding parameters from the global fit of the full time courses and activity-based binding experiments are within 2-fold of those determined previously (11, 12, 17, 18). The bimolecular rate constants for Pm generation by the SK-Pg' and SK-Pm catalytic complexes previously determined were 0.5 ± 0.05 and 1.16 ± 0.04 μM\(^{-1}\) s\(^{-1}\), respectively, whereas here they were 1.17 ± 0.17 and 2.4 ± 0.4 μM\(^{-1}\) s\(^{-1}\), respectively. The reason for this relatively small difference is not known, but it may be due to differences in the assumptions made here for the discontinuous assays and those in the previous studies from analysis of continuous parabolic assays in the presence of chromogenic substrate.

Specific molecular events are thought to dictate the sequential pathway of [Lys]Pg activation by SK. The trigger catalytic cycle depends on (a) conformational rearrangement of the disordered domain structure of SK in solution into the ordered structure it forms on binding of Pg and plasmin seen in the SK-Pm crystal structure (5, 16); (b) induced conformational activation of Pg by SK NH\(_2\)-terminal insertion (7–9); (c) engagement of the LBS of a Pg kringle, probably kringle 4, by SK Lys\(^{414}\) that increases affinity of SK-Pg* formation (13, 45); (d) expression of the exosite on SK-Pg* that mediates Pg substrate binding, as shown for Pm (11); and (e) engagement of LBS interactions with kringle 5 mediated by Arg\(^{252}\), Lys\(^{254}\), and Lys\(^{257}\) in the SK β-domain that enhance Pg substrate binding (20). Although required, the order in which events c, d, and e occur has not been completely defined.

The second, bullet cycle depends on intermolecular cleavage of Pg bound to SK-Pg* in the substrate mode to form Pm in the trigger cycle. Free Pm and free Pg bind competitively to SK in the catalytic mode. Pm has the advantage over Pg in competitive catalytic complex formation, binding SK with 500–900-fold higher affinity than [Lys]Pg, which results in transition of the catalytic complexes from SK-Pg* to SK-Pm as the reactions proceed. This transition is important because it is at the junction of the catalytic cycles. Because Assay 1 contained 10 μM 6-AHA, the affinity difference between [Lys]Pg and Pm for SK was reduced to ∼30-fold, nevertheless sufficient to support transition to the SK-Pm complex. Once sufficient Pm is produced to bind all of the SK, the trigger cycle stops, and SK-Pm is the only catalytic complex. SK-Pm binding of Pg as substrate is also thought to be facilitated by the same residues in the β-domain used by SK-Pg* (20) and produces Pm at a ∼2-fold faster bimolecular rate constant than SK-Pg*, ultimately converting all of the remaining Pg to Pm. The off-rate constant for dissociation of the SK-Pm complex is ∼0.0008 s\(^{-1}\) (\( t_{1/2} \sim 900 \) s (45)), suggesting that the SK-Pm complex may not dissociate significantly during the full time course of the bullet catalytic cycle.

Rapid reaction kinetics of SK binding to active site-labeled Pm demonstrate a three-step mechanism of rapid encounter complex formation followed by two sequential affinity-enhancing conformational changes (45). Comparison of the effects of kringle ligands and a SK Lys\(^{414}\) deletion mutant identified the encounter complex as the primary source of the LBS dependence of SK-Pm catalytic complex formation, whereas the two conformational changes are less affected. The first step in the binding pathway requires on-rate constants of ∼1 nm\(^{-1}\) s\(^{-1}\), indicating near-diffusion controlled encounter complex formation (45). Remarkably, the fitted rate constant for SK-Pm formation determined here was 1.2 nm\(^{-1}\) s\(^{-1}\) (Table 1). This suggests that the initial step in the SK-Pm catalytic cycle is fast compared with the fitted value of 0.1 nm\(^{-1}\) s\(^{-1}\) for SK-Pg*, and the engagement of Lys\(^{414}\) with a Pm kringle occurs before the tightening conformational changes of SK-Pm.

Concerning the role of SK as a virulence factor in streptococcal infections, generation of Pm is critical for dissemination of the bacteria through tissues by directly cleaving extracellular matrix proteins, indirectly activating metalloproteinases, and dissolving fibrin barriers established in the initial host response to infection (52–55). The SK-Pg activation mechanism is regulated by fibrinogen and fibrin through a SK-Pg-fibrinogen(ogen) ternary complex and by Pg-binding group A streptococcal M-like protein (PAM) that is covalently bound to the bacterial cell wall (46–50, 53, 54, 56).

PAM mediates Pg and Pm binding through two type a, continuous repeat sequences near the NH\(_2\) terminus of its α-helical, dimeric coiled-coil structure, which extends into solution about 500 Å from the LPXTG motif near the COOH terminus.
that links PAM to the cell wall (57–59). Direct binding of Pm or SK-Pg*-generated Pm bound to PAM results in coating of the bacterial surface by proteolytically active PAM-Pm complexes that are resistant to inactivation by AP, allowing the bacteria to spread rapidly through fibrin barriers and the extracellular matrix like proteolytic chain saws. Other members of the M protein superfamily lack the Pm-binding motifs, but a subset of them, including M1, bind Fbg specifically through the b-type repeats (53, 54, 57, 58, 60). This begins what is called the indi-

cates the mechanisms of allelic variants of SK (3, 4, 62) and other pathogenic effectors of SK-Pg activation.

REFERENCES

1. Carapetis, J. R., Steer, A. C., Mulholland, E. K., and Weber, M. (2005) The global burden of group A streptococcal diseases. Lancet Infect. Dis. 5, 685–694

2. Timoney, J. F. (2004) The pathogenic equine streptococcus. Vet. Res. 35, 397–409

3. Cook, S. M., Skora, A., Gillen, C. M., Walker, M. J., and McArthur, J. D. (2012) Streptokinase variants from Streptococcus pyogenes isolates display altered plasminogen activation characteristics. Implications for pathogenesis. Mol. Microbiol. 86, 1052–1062

4. Kalia, A., and Bessen, D. E. (2004) Natural selection and evolution of streptococcal virulence genes involved in tissue-specific adaptations. J. Bacteriol. 186, 110–121

5. Wang, X., Lin, X., Loy, J. A., Tang, J., and Zhang, X. C. (1998) Crystal structure of the catalytic domain of human plasmin complexed with streptokinase. Science 281, 1662–1665

6. Bode, W., and Huber, R. (1976) Induction of the bovine trypsinogen-
trypsin transition by peptides sequentially similar to the N-terminus of trypsin. FEBS Lett. 68, 231–236

7. Wang, S., Reed, G. L., and Hedstrom, L. (1999) Deletion of Ile1 changes the mechanism of streptokinase. Evidence for the molecular sexuality hypothesis. Biochemistry 38, 5232–5240

8. Wang, S., Reed, G. L., and Hedstrom, L. (2000) Zymogen activation in the streptokinase-plasminogen complex. Ile1 is required for the formation of a functional active site. Eur. J. Biochem. 267, 3994–4001

9. Boxrud, P. D., Verhamme, I. M., Fay, W. P., and Bock, P. E. (2001) Streptokinase triggers conformational activation of plasminogen through specific interactions of the amino-terminal sequence and stabilizes the active zymogen conformation. J. Biol. Chem. 276, 26084–26089

10. Friedrich, R., Panizzi, P., Fuentes-Prior, P., Richter, K., Verhamme, I., Anderson, P. J., Kawabata, S., Huber, R., Bode, W., and Bock, P. E. (2003) Staphylococcal agglutinase is a prototype for the mechanism of cofactor-induced zymogen activation. Nature 425, 535–539

11. Boxrud, P. D., Fay, W. P., and Bock, P. E. (2000) Streptokinase binds to human plasmin with high affinity, perturbs the plasmin active site, and induces expression of a substrate recognition exosite for plasminogen. J. Biol. Chem. 275, 14579–14589

12. Boxrud, P. D., and Bock, P. E. (2000) Streptokinase binds preferentially to the extended conformation of plasminogen through lysine binding site and catalytic domain interactions. Biochemistry 39, 13974–13981

13. Panizzi, P., Boxrud, P. D., Verhamme, I. M., and Bock, P. E. (2006) Binding of the COOH-terminal lysine residue of streptokinase to plasminogen (ogen) kringles enhances formation of the streptokinase-plasminogen (ogen) catalytic complexes. J. Biol. Chem. 281, 26774–26778

14. Wohl, R. C., Summara, L., Arzadon, L., and Robbins, K. C. (1978) Steady state kinetics of activation of human and bovine plasminogens by streptokinase and its equimolar complexes with various activated forms of human plasminogen. J. Biol. Chem. 253, 1402–1407

15. Wohl, R. C., Summara, L., and Robbins, K. C. (1980) Kinetics of activation of human plasminogen by different activator species at pH 7.4 and 37 °C. J. Biol. Chem. 255, 2005–2013

16. Damaschun, G., Damaschun, H., Gast, K., Gerlach, D., Misset, W., Wedde, H., and Zirwer, D. (1992) Streptokinase is a flexible multi-domain protein. Eur. Biophys. J. 20, 355–361

17. Boxrud, P. D., Verhamme, I. M., and Bock, P. E. (2004) Resolution of conformational activation in the kinetic mechanism of plasminogen activation by streptokinase. J. Biol. Chem. 279, 36633–36641

18. Boxrud, P. D., and Bock, P. E. (2004) Coupling of conformational and proteolytic activation in the kinetic mechanism of plasminogen activation by streptokinase. J. Biol. Chem. 279, 36642–36649

19. Joshi, K. K., Nanda, J. S., Kumar, P., and Sahnhi, G. (2012) Substrate kringle-mediated catalysis by the streptokinase-plasmin activator complex. Critical contribution of kringle-4 revealed by the mutagenesis approaches. Biochim. Biophys. Acta 1824, 326–333

20. Tharp, A. C., Laha, M., Panizzi, P., Thompson, M. W., Fuentes-Prior, P., and Bock, P. E. (2009) Plasminogen substrate recognition by the streptokinase-plasminogen catalytic complex is facilitated by Arg15 and Lys256, and Lys257 in the streptokinase β-domain and kringle 5 of the substrate. J. Biol. Chem. 284, 19511–19521

21. Chaudhary, A., Vasudha, S., Rajagopal, K., Komath, S. S., Garg, N., Yadav, M., Mande, S. C., and Sahnhi, G. (1999) Function of the central domain of streptokinase in substrate plasminogen docking and processing revealed by site-directed mutagenesis. Protein Sci. 8, 2791–2805

22. Dhar, J., Pande, A. H., Sundram, V., Nanda, J. S., Mande, S. C., and Sahnhi, G. (2002) Involvement of a nine-residue loop of streptokinase in the generation of macromolecular substrate specificity by the activator complex through interaction with substrate kringle domains. J. Biol. Chem. 277, 13257–13267

23. Bock, P. E., Day, D. E., Verhamme, I. M., Bernardo, M. M., Olson, S. T., and Shore, J. D. (1996) Analogs of human plasminogen that are labeled with fluorescence probes at the catalytic site of the zymogen. Preparation, characterization, and interaction with streptokinase. J. Biol. Chem. 271, 1072–1080

24. Deutsch, D. G., and Mertz, E. T. (1970) Plasminogen. Purification from human plasma by affinity chromatography. Science 170, 1095–1096

25. Dharmawardana, K. R., and Bock, P. E. (1998) Demonstration of exosite I-dependent interactions of thrombin with human factor V and factor Va involving the factor Va heavy chain. Analysis by affinity chromatography employing a novel method for active-site-selective immobilization of serine proteinases. Biochemistry 37, 13143–13152

26. Nieuwenhuizen, W., and Tras, D. W. (1989) A rapid and simple method for the separation of four molecular forms of human plasminogen. Thromb. Haemost. 61, 208–210

27. Castellino, F. J., and Powell, I. R. (1981) Human plasminogen. Methods Enzymol. 80, 365–378

28. Sjo¨holm, I. (1973) Studies on the conformational changes of plasminogen induced during activation to plasmin and β-aminohexanoic acid. Eur. J. Biochem. 39, 471–479

29. Violand, B. N., and Castellino, F. J. (1976) Mechanism of the urokinase-catalyzed activation of human plasminogen. J. Biol. Chem. 251, 3906–3912

30. Jackson, K. W., and Tang, J. (1982) Complete amino acid sequence of streptokinase and its homology with serine proteinases. Biochemistry 21, 6620–6625

31. Wilmam, B., and Collen, D. (1977) Purification and characterization of human antiplasmin, the fast-acting plasmin inhibitor in plasma. Eur. J. Biochem. 78, 19–26

32. Moroi, M., and Aoki, N. (1976) Isolation and characterization of α2-plasmin inhibitor from human plasma. A novel proteinase inhibitor which inhibits activator-induced clot lysis. J. Biol. Chem. 251, 5956–5965

33. Engvall, E., and Ruoslahti, E. (1977) Binding of soluble form of fibroblast
surface protein, fibronectin, to collagen. *Int. J. Cancer* **20**, 1–5
34. Naski, M. C., and Shafer, J. A. (1991) A kinetic model for the α-thrombin-catalyzed conversion of plasma levels of fibrinogen to fibrin in the presence of antithrombin III. *J. Biol. Chem.*** **266**, 13003–13010
35. Hogg, P. I., and Jackson, C. M. (1990) Heparin promotes the binding of thrombin to fibrin polymer. Quantitative characterization of a thrombin-fibrin polymer-heparin ternary complex. *J. Biol. Chem.*** **265**, 241–247
36. Weisel, J. W. (2005) Fibrinogen and fibrin. in *Advances in Protein Chemistry* (David, A. D. P., and John, M. S., eds) pp. 247–299, Academic Press, Inc., New York
37. Lottenberg, R., Hall, J. A., Blinder, M., Binder, E. P., and Jackson, C. M. (1983) The action of thrombin on peptide p-nitroanilide substrates. Substrate selectivity and examination of hydrolysis under different reaction conditions. *Biochim. Biophys. Acta*** **742**, 539–557
38. Lottenberg, R., and Jackson, C. M. (1983) Solution composition dependent variation in extinction coefficients for p-nitroaniline. *Biochim. Biophys. Acta*** **742**, 558–564
39. Alkaersig, N., Fletcher, A. P., and Sherry, S. (1959) É-Aminocaproic acid. An inhibitor of plasminogen activation. *J. Biol. Chem.*** **234**, 832–837
40. Lin, L. F., Houng, A., and Reed, G. L. (2000) ÿ amino caproic acid inhibits streptokinase-plasminogen activator complex formation and substrate binding through kringle-dependent mechanisms. *Biochemistry*** **39**, 4740–4745
41. Cederholm-Williams, S. A., De Cock, F., Lijnen, H. R., and Collen, D. (1979) Kinetics of the reactions between streptokinase, plasmin and ñ-antiplasmin. *Eur. J. Biochem.*** **100**, 125–132
42. Lijnen, H. R., Van Hoef, B., De Cock, F., Okada, K., Ueshima, S., Matsuoi, O., and Collen, D. (1991) On the mechanism of fibrin-specific plasminogen activation by staphylokinase. *J. Biol. Chem.*** **266**, 11826–11832
43. Johnson, K. A., Simpson, Z. B., and Blom, T. (2009) Global Kinetic Explorer. A new computer program for dynamic simulation and fitting of kinetic data. *Anal. Biochem.*** **387**, 20–29
44. Johnson, K. A., Simpson, Z. B., and Blom, T. (2009) FitSpace Explorer. An algorithm to evaluate multidimensional parameter space in fitting kinetic data. *Anal. Biochem.*** **387**, 30–41
45. Verhamme, I. M., and Bock, P. E. (2008) Rapid-reaction kinetic characterization of the pathway of streptokinase-plasmin catalytic complex formation. *J. Biol. Chem.*** **283**, 26137–26147
46. Chibber, B. A., Morris, J. P., and Castellino, F. J. (1985) Effects of human fibrinogen and its cleavage products on activation of human plasminogen by streptokinase. *Biochemistry*** **24**, 3429–3434
47. Strickland, D. K., Morris, J. P., and Castellino, F. J. (1982) Enhancement of the streptokinase-catalyzed activation of human plasminogen by human fibrinogen and its plasminolysis products. *Biochemistry*** **21**, 721–728
48. Wang, H., Lottenberg, R., and Boyle, M. D. (1995) Analysis of the interaction of group A streptococci with fibrinogen, streptokinase and plasminogen. *Microb. Pathog.*** **18**, 153–166
49. Takada, A., Takada, Y., and Sugawara, Y. (1985) The activation of Glu- and Lys-plasminogens by streptokinase. Effects of fibrin, fibrinogen and their degradation products. *Thromb. Res.*** **37**, 465–475
50. Takada, Y., and Takada, A. (1989) Evidence for the formation of a trimolecular complex between streptokinase, plasminogen and fibrinogen. *Thromb. Res.*** **53**, 409–415
51. Hess, J. L., and Boyle, M. D. (2006) Fibrinogen fragment D is necessary and sufficient to anchor a surface plasminogen-activating complex in *Streptococcus pyogenes*. *Proteomics*** **6**, 375–378
52. Sun, H. (2006) The interaction between pathogens and the host coagulation system. *Physiology*** **21**, 281–288
53. Sanderson-Smith, M. L., Dinkla, K., Cole, J. N., Cork, A. I., Maamary, P. G., McArthur, J. D., Chhatwal, G. S., and Walker, M. J. (2008) M protein-mediated plasminogen binding is essential for the virulence of an invasive *Streptococcus pyogenes* isolate. *FASEB J.* **22**, 2715–2722
54. Walker, M. J., McArthur, J. D., McKay, F., and Ranson, M. (2005) Is plasminogen deployed as a *Streptococcus pyogenes* virulence factor? *Trends Microbiol.* **13**, 308–313
55. Sun, H., Ringdahl, U., Homeister, J. W., Fay, W. P., Engleberg, N. C., Yang, A. Y., Roze, L., Wang, X., Sjöbring, U., and Ginsburg, D. (2004) Plasminogen is a critical host pathogenicity factor for group A streptococcal infection. *Science*** **305**, 1283–1286
56. Berge, A., and Sjöbring, U. (1993) PAM, a novel plasminogen-binding protein from *Streptococcus pyogenes*. *J. Biol. Chem.*** **268**, 25417–25424
57. Macheboeuf, P., Buffalo, C., Fu, C. Y., Zinkernagel, A. S., Cole, J. N., Johnson, J. E., Nizet, V., and Ghosh, P. (2011) Streptococcal M1 protein constructs a pathological host fibrinogen network. *Nature*** **472**, 64–68
58. McNamara, C., Zinkernagel, A. S., Macheboeuf, P., Cunningham, M. W., Nizet, V., and Ghosh, P. (2008) Coiled-coil irregularities and instabilities in group A *Streptococcus* M1 are required for virulence. *Science*** **319**, 1405–1408
59. Phillips, G. N., Jr., Flicker, P. F., Cohen, C., Manjula, B. N., and Fischetti, V. A. (1981) Streptococcal M protein. ñ-Helical coiled-coil structure and arrangement on the cell surface. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4689–4693
60. Sanderson-Smith, M. L., Walker, M. J., and Ranson, M. (2006) The maintenance of high affinity plasminogen binding by group A streptococcal plasminogen-binding M-like protein is mediated by arginine and histidine residues within the a1 and a2 repeat domains. *J. Biol. Chem.*** **281**, 25965–25971
61. Boyle, M. D., and Lottenberg, R. (1997) Plasminogen activation by invasive human pathogens. *Thromb. Haemost.* **77**, 1–10
62. Zhang, Y., Liang, Z., Hsueh, H.-T., Plophuis, V. A., and Castellino, F. J. (2012) Characterization of streptokinases from group A streptococci reveals a strong functional relationship that supports the coinherance of plasminogen-binding M protein and cluster 2b streptokinase. *J. Biol. Chem.*** **287**, 42093–42103