Coupling of Conformational and Proteolytic Activation in the Kinetic Mechanism of Plasminogen Activation by Streptokinase*

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Binding of streptokinase (SK) to plasminogen (Pg) induces conformational activation of the zymogen and initiates its proteolytic conversion to plasmin (Pm). The mechanism of coupling between conformational activation and Pm formation was investigated in kinetic studies. Parabolic time courses of Pg activation by SK monitored by chromogenic substrate hydrolysis had initial rates (v1) representing conformational activation and subsequent rates of activity increase (v2) corresponding to the rate of Pm generation determined by a specific discontinuous assay. The v2 dependence on SK concentration for [Lys]Pg showed a maximum rate at a Pg to SK ratio of ~2:1, with inhibition at high SK concentrations.

[Glut]Pg and [Lys]Pg activation showed similar kinetic behavior but much slower activation of [Glut]Pg, due to an ~12-fold lower affinity for SK and an ~20-fold lower kcat/Km. Blocking lysine-binding sites on Pg inhibited SK/Pg cleavage of [Lys]Pg to a rate comparable with that of [Glut]Pg, whereas [Glut]Pg activation was not significantly affected. The results support a kinetic mechanism in which SK activates Pg conformationally by rapid equilibrium formation of the SK-Pg complex, followed by intermolecular cleavage of Pg to Pm by SK-Pg and subsequent cleavage of Pg by SK-Pm. A unified model of SK-induced Pg activation suggests that generation of initial Pm by SK-Pg acts as a self-limiting triggering mechanism to initiate production of one SK equivalent of SK-Pm, which then converts the remaining free Pg to Pm.

Streptokinase (SK) is a protein from Streptococcus equisimilis that converts the zymogen, plasminogen (Pg), into the fibrin clot-dissolving serine proteinase, plasmin (Pm) (1). The domain organization of the circulating form of Pg ([Glu]Pg) consists of a 77-residue N-terminal peptide, five kringle domains, and a serine proteinase catalytic domain that is activated by cleavage at Arg561–Val562 (2, 3). Pm cleavage of [Glu]Pg releases the N-terminal peptide, yielding [Lys]Pg, which is more readily activated by plasminogen activators, including SK-Pg and SK-Pm (4–11). The mechanism of Pg activation by SK is unique in that SK binding to Pg results in expression of an active catalytic site on the zymogen by a conformational change, without the normally required peptide bond cleavage (12, 13). Conformational activation is followed by proteolytic conversion of Pg to Pm by cleavage of Arg561–Val562, and SK bound to Pm propagates proteolytic Pg activation. Recognition of Pg as a substrate of SK-Pm is mediated by expression of a specific substrate recognition exosite on the SK-Pm complex (14, 15).

Both conformational and proteolytic activation reactions are known to contribute to SK-induced Pg activation, but different interpretations of the mechanism of conformational activation and its coupling to subsequent proteolytic Pm formation have been postulated. Quantitative equilibrium binding and kinetic analysis of conformational activation in the companion paper (16) demonstrated that it can be described by rapid and reversible SK binding and activation of the catalytic site. The activation equilibrium is more favorable for Pg species with an extended conformation, [Lys]Pg and [Glu]Pg at low chloride concentration (4, 5, 17), compared with [Glu]Pg in the compact conformation (11, 18, 19), due to the differential involvement of lysine-binding site interactions.

Previous steady-state kinetic studies of SK-initiated Pm formation concluded that Pm was formed initially by intramolecular proteolytic activation of Pg within the conformationally activated SK-Pg complex (20). The subsequent findings that SK-Pm did not catalyze a mixture of SK, Pg, and bovine trypsin inhibitor to Pm and the resistance of diisopropyl fluorophosphate-inactivated SK-Pg to Pg activators also supported the intramolecular cleavage hypothesis (21, 22). More recent studies (14, 23, 24), however, provide evidence for an SK-Pg**Pm ternary complex, suggesting that intermolecular cleavage of Pg bound to SK-Pg** in a substrate mode contributes to initial Pm formation. Equilibrium binding studies demonstrated that the reaction product [Lys]Pg binds SK with ~50,000- and ~11,000-fold higher affinity to fluorescein-labeled Pg analogs (15, 25–27), and native Pg (16), respectively, compared with the initial substrate, [Glu]Pg. The large difference in affinity may result in displacement of Pg from the SK-Pg** complex by Pm, forming the very stable proteolytic Pg activator complex SK-Pm. The pathway of Pm generation from SK-Pg** and the source of SK-Pm are unclear, in part because of conflicting evidence for the intramolecular and intermolecular mechanisms.

The role of lysine-binding sites on the kringle domains of Pg and Pm in the mechanism of SK-induced Pg activation is also only partly understood. The lysine analog, 6-aminoheptonic acid (6-AHA), partially reduces binding affinity of SK for [Lys]Pg in parallel with inhibition of conformational activation,
but does not affect [Glu]Pg (16, 25, 26), and inhibits overall formation of Pm (28, 29). It appears that the affinity of SK binding to Pg and subsequent proteolytic formation of Pm are both inhibited by 6-AHA, but the relationship between these sources of inhibition and the basis for the differential effect on [Glu]Pg and [Lys]Pg activation are not clear.

The goal of the present studies was to apply the results of previous equilibrium binding and kinetic studies of SK-Pg and SK-Pm interactions in combination with initial rate kinetic analysis to define the coupling of conformational Pg activation to the proteolytic activation pathway of SK-induced Pm formation. The studies address the unresolved mechanism of proteolytic Pm formation, the relationship between catalysis by SK-Pg* and SK-Pm complexes, and regulation of these events by lysine-binding site interactions. The kinetics of proteolytic Pg activation and inhibition of Pm formation by saturating SK concentrations observed here is explained by a mechanism in which Pm is formed by intermolecular cleavage of Pg by the SK-Pg* complex. The binding and conformational activation equilibrium for [Lys]Pg, characterized in the companion paper (16), is partially facilitated by lysine-binding site interactions, whereas subsequent Pm formation is greatly dependent on these interactions. By contrast, the independence of [Glu]Pg conformational and proteolytic activation on lysine-binding sites accounts for the higher substrate specificity of the SK-Pg* complex for [Lys]Pg compared with [Glu]Pg. The combined equilibrium binding and kinetic data do not support a significant role for intramolecular Pm formation within the SK-Pg* complex. The studies provide the first quantitative kinetic analysis of the pathway of Pm formation by SK and demonstrate the critical role of differences in SK affinity for Pg activation species and the differential roles of lysine-binding site interactions in initiating and propagating the activation mechanism. A unified kinetic mechanism of Pg activation suggests that the activation process at concentrations of Pg >> SK is triggered by a self-limiting initial cycle of Pm formation catalyzed by SK-Pg* that results in sequestering of SK in the SK-Pm complex, which ultimately catalyzes full conversion of free Pg to Pm.

**Scheme 1**

\[
\text{SK} + \text{Pg} \leftrightarrow K_a \text{SKP-g* + Pg} \leftrightarrow K_0 \text{SKP-g*Pg} \rightarrow K_0 \text{SKP-g* + Pm} + S \\
S + K_m \rightarrow \text{SKP-g}*S \\
\text{SKP-g}*S \rightarrow K_m \text{SKP-g* + Pm} + S \\
\text{SKP-g* + Pm} \rightarrow K_c \text{Pm + P} + S
\]

**Scheme 2**

\[
\text{SK} + \text{Pm} \leftrightarrow K_a \text{SKP-g* + Pm} \leftrightarrow K_0 \text{SKP-g*Pm} \rightarrow K_0 \text{SKP-g* + Pm} + S \\
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## Experimental Procedures

**Protein Purification and Characterization**—[Glu]Pg, [Lys]Pg, Pm, and SK were prepared and their concentrations determined as described in the companion paper (16).

**Gel Electrophoresis**—Pg activation reactions were initiated at 25 °C by addition of SK in 50 mM Hepes, 0.125 mM NaCl, 1 mM EDTA, 1 mg/ml polyethylene glycol 8000, pH 7.4, and inactivated with 100 μM (D-Phe)-Phe-Arg-CH₂Cl for 2 min at room temperature before preparation of gel samples and electrophoresis on SDS 4−15% polyacrylamide gels. Gels were stained with Sypro Orange (Molecular Probes) and protein bands visualized with a 300-nm transilluminator.

**Plasminogen Activation Kinetics**—Activation of Pg by SK was measured by continuous monitoring of the increase in absorbance of o-Val-Leu-Lys-pNA (VLK-pNA) at 405 nm (ΔA₄₀₅ nm). The parabolic progress curves of Pm formation were fit by Equation 1 (30, 31) to obtain the initial rate of substrate hydrolysis at the beginning of the reaction (v₁) and the rate of increase in activity with time (v₂).

\[
\Delta A_{405 \text{ nm}} = \frac{1}{2} v_1 t + v_2 t
\]

Linear transformation of the time courses was done by dividing ΔA₄₀₅ nm by t (20). Michaelis-Menten parameters for SK-Pm-catalyzed activation of [Glu]Pg and [Lys]Pg were determined from the hyperbolic dependences of v₁ on the total Pg concentration at 0.1 nM SK and 0.5 nM Pm. Because of the very low dissociation constant (12 μM), v₁ corresponded to 0.1 nM SK-Pm complex. Free Pm and SK-Pm contributed to v₁, but Pm alone did not activate Pg (15, 31).

**Discontinuous Plasmin Assay**—Discontinuous assays of the initial rates of Pm generation in reactions of Pg and SK were performed by incubation of SK and [Lys]Pg for various times and quenching the reaction by addition of 1 μM FFR-Pm and 10 mM 6-AHA to dissociate the SK-Pg* complex and to inhibit additional formation of Pm. Following 2 min of incubation, chromogenic substrate assays of quenched samples were initiated by addition of 50 μM VLK-pNA and the concentrations of Pm were calculated from slopes of the linear rates. The substrate concentration was chosen such that free Pm and SK-bound Pm had equal contributions to the rate, independent of SK concentration (15). Discontinuous assays of Pm generation were linear with time at up to 60% Pg depletion, and data were fit by linear least squares to determine the rates. Pre-quenched reactions in which 6-AHA and FFR-Pm were present in the incubation resulted in rates of essentially zero. The activities of quenched reactions were stable with time, exhibiting <0.5% increase in activity over 20 min after quenching. The assay yielded the Pm concentration accurately in mixtures of Pg or SK containing known levels of Pm.

**Kinetic Model of Plasminogen Activation by Streptokinase**—The dependences of v₁ and v₂ on SK concentration can be described by the mechanism in Schemes 1 and 2. In Scheme 1, SK binds to Pg with dissociation constant Kₐ to form the conformationally activated SK-Pg* complex, which catalyzes chromogenic substrate (S) hydrolysis with Michaelis-Menten parameters Kₐ and kₐ. Binding of Pm as a substrate of SK-Pg* with dissociation constant Kₐ forms the ternary Michaelis complex (SK-Pg*Pm) that generates Pm with the catalytic rate constant kₐ. As illustrated in Scheme 2, the SK-Pm complex can also catalyze Pg activation to Pm, and SK-Pm hydrolyzes the chromogenic substrate with Michaelis constants Kₐ and kₐ. The extremely high affinity of SK for Pm (Kₐ < 12 μM) compared with [Glu]Pg and [Lys]Pg is assumed to result in SK-Pm as the product. Under initial conditions where [SK]₀ > [Pm]₀, all of the Pm generated is present as SK-Pm, whereas at [SK]₀ < [Pm]₀, all SK becomes complexed with Pm as the reaction progresses. With the assumption that Pg activation by SK-Pm (Scheme 2) does not contribute significantly to the initial rate, and only modestly in reactions with [Lys]Pg at [SK]₀ < [Pm]₀, the observed progress of activity formation is given by Equation 1, where v₁ is described by the equations in the companion paper (16), and the initial rate of Pm generation by the SK-Pg* complex (v₂) is given by Equation 2.

\[
v_2 = k_o [\text{SKP-g*Pg}]
\]

Dividing v₂ by [SK]₀ in terms of [P_m]ₜₚₑₑ gives Equation 3,

\[
v_2 = \frac{k_o [\text{P_m}]_{\text{tot}}}{K_m (1 + [S]_0 + [P_m]_{\text{tot}})/[SK]₀ + [P_m]_{\text{tot}}}
\]

where [P_m]ₜₚₑₑ is given by the cubic equation described previously (16). Under conditions where [P_m]ₜₚₑₑ ≪ Kₐ and [S]₀ ≪ Kₐ, which were
both proteolytic reactions in Schemes 1 and 2, Reactions 1–6 were simulated by numerical integration of the differential rate equations that the proteolytic reaction in Scheme 2 could also be represented as desired dissociation constants, where reverse rate constants were assigned appropriate values to give the for the SK/H18528 Pm complex with rate constant SK rate constant /H18528 K were calculated by using described in the companion paper (16). 

As will be shown, the concentrations of Pg were also much lower than K_a for activation of Pg as a substrate of the SK/H18528 Pm complex, indicating the proteolytic activation step can be treated as bimolecular with rate constant k_{Pm} found to apply (16), Equation 3 can be reduced to Equation 4, and the proteolytic activation progress curves as a function of SK and Pg concentrations were calculated as 

\[ v_2 = \frac{k_{Pg}[SK]_0[Pg]_{free}}{1 + \frac{K_A}{[Pg]_{free}}} \]  

(Eq. 4) 

where [Pg]_{free} is defined by the quadratic equation in [Pg], and [SK]_0 described in the companion paper (16). v_2 rates expressed as [Pm] s^{-1} were calculated by using K_{pm} = 300 \mu M and k_1' = 34 s^{-1} as determined for the SK/Pm complex (15). The v_2 dependencies were obtained from activation progress curves as a function of SK and Pg concentrations analyzed by fitting the simplified Equation 4 and the quadratic binding equation.

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Proteolytic Plasminogen Activation by Streptokinase

In this scheme, SK binds to Pg with the dissociation constant $K_A$, forming the conformationally activated SK-Pg* complex, which subsequently binds and cleaves Pg to Pm with bimolecular rate constant $k_{p_m}$. Results in the companion paper (16) demonstrated that representation of the formation of SK-Pg* as a single binding and activation equilibrium, and the SK-Pg*-catalyzed proteolytic reaction as bimolecular were justified. $v_2$ values from continuous assays of reactions of 2–20 nm [Lys]Pg1 with varying SK (Fig. 3A) were fit by Equation 4 and the quadratic binding equation derived for Scheme 3 with the assumption that $[Pg^*_act] \ll K_{p_m}$ and $[Pg^*]_0 \ll K_{p_m}$, as described under "Experimental Procedures." Analysis of the dependence of $v_1$ on SK concentration gave a good fit to the data with 12 ± 3 nm for $K_A$ (16). Simultaneous analysis of the $v_2$ data resulted in a value of 2.3 ± 0.2 nm for $K_{p_m}$, which was 5-fold lower than the value obtained from the $v_1$ data and $k_{p_m}$ of 0.79 ± 0.02 µM$^{-1}$ s$^{-1}$ (Fig. 3A). Comparable values were obtained for reactions of 5–20 nm [Lys]Pg1 with SK (Table 1).

Similar experiments performed with [Glu]Pg showed much slower reactions and less inhibition by excess SK. Fits of $v_1$ from continuous assay titrations of 5–20 nm [Glu]Pg1 yielded 143 ± 12 nm for $K_A$ (16), whereas analysis of the $v_2$ data resulted in $K_A$ of 414 ± 57 nm and $k_{p_m}$ of 0.04 ± 0.01 µM$^{-1}$ s$^{-1}$ (Fig. 3B and Table I). These fits demonstrated weaker binding of SK to [Glu]Pg compared with [Lys]Pg, as indicated in direct binding studies (16), and a 20-fold reduction in Pg generation measured by $k_{p_m}$ (Table I).

6-AHA decreased $v_2$ for [Lys]Pg activation dramatically (Fig. 4), by ~50-fold compared with the maximum rate, resulting in more linear progress curves at all SK concentrations. The $v_2$ values resulting from activation of [Glu]Pg, in contrast to [Lys]Pg activation, were not affected significantly by 6-AHA and were comparable with those of [Lys]Pg in 6-AHA (Fig. 4, inset). These results indicated that inhibition of Pg formation by 6-AHA was due to both partial reduction of the affinity of SK for [Lys]Pg and greater inhibition of subsequent proteolytic activation. By contrast, the affinity of SK for [Glu]Pg and its proteolytic conversion to Pm were not significantly lysine-binding site-dependent.
Simulation of the Reaction Mechanism—The major assumption in the above analysis was that SK-Pm-catalyzed Pg activation (Scheme 2, see “Experimental Procedures”) did not contribute significantly to the initial rate of Pm formation. This was examined further by simulation of the coupled SK-Pg* and SK-Pm reactions in Scheme 3 by numerical integration of the differential rate equations. The kinetic parameters for SK-Pm-catalyzed cleavage of [Lys]Pg (K_S and k_o in Scheme 2) were determined in v_2 measurements of [Lys]Pg activation by SK-Pm formed with an excess of Pm (data not shown), where Pm alone does not activate Pg (15, 31). K_S and k_o for this reaction were 270 ± 60 nM and 0.31 ± 0.02 s⁻¹ (Table I). K_S was >13-fold higher than the highest Pg concentration used experimentally (Fig. 3A), allowing the proteolytic reaction to be treated as bimolecular with rate constant k_pm as shown in Scheme 3. For the simulations, the bimolecular rate constants k_pg and k_pm and the dissociation constant for the SK-Pm complex (K_A) were fixed at their determined values, whereas K_A was assigned a value of 4.5 nM, comparable with values determined for [Lys]Pg from analysis of v_1 (16) and here for v_3 (2–12 nM). These parameters gave the dashed line in Fig. 1A for the time course of the increase in total Pm concentration, which agreed well with the rates measured by the continuous and discontinuous v_2 assays. Simulation of the data under the conditions of Fig. 1A showed that the linear rate was a very sensitive function of K_A, with the best fit by a value 4.5 nM.

Given the sequential nature of the SK-Pg* and SK-Pm-catalyzed Pg activation reactions, it was unclear why the progress curves of Pm formation were apparently linear over a wide range of SK and [Lys]Pg concentrations and for up to 50–60% substrate depletion. Simulation of the time courses of the appearance and disappearance of the reaction species at 10 nM [Lys]Pg and substoichiometric 5 nM SK with the above parameters gave the results shown in Fig. 5A, where α represents free PmA formed by SK-Pg* and β represents SK-PmA. The total PmA concentration, free and SK-bound (Fig. 5A, α + β), increased to a steady maximum, whereas the total Pmb concentration (c + d) showed a lag, with an increase to a near-linear rate that was initially accounted for primarily by formation of SK-PmB (Fig. 5A, d), consistent with the sequential reactions. The total Pm concentration increased, initially following the rate of PmA formation, and curved upward slightly as Pmb was produced (Fig. 5A, PmB). Linear least squares analysis of the simulated initial rate of PmB formation demonstrated that it represented the rate of total PmA formation, as expected from the sequential reactions. Similar linear analysis of the rate of PmB formation at [SK]_0 < [Pg]_0 for up to 50% Pg depletion gave rates that were overestimated by a maximum of 2-fold compared with the initial rates calculated with Equation 4. These rates corresponded closely to the values of v_2 determined experimentally (Fig. 3A).

Simulation of the reaction time course at excess SK concentration (50 nM) with the same parameters gave the results in Fig. 5B. Under these reaction conditions, the rate of total Pm formation (Fig. 5B, Pm_T) was quite linear and agreed well with the initial rate calculated for the increase in total PmA concentration (α + β) from Equation 4. Analysis of the reaction products with increasing SK concentration indicated that inhibition of the rate by excess SK was due to depletion of the free Pg by formation of the SK-Pg* complex, with inhibition of the SK-Pm-catalyzed reaction for the same reason. It was apparent from these results that the modest upward curvature in the rate of total Pm generation from [Lys]Pg at substoichiometric levels of SK would not have been detectable in the experimental progress curves. It was also clear that to within a factor of 2-fold, the linear approximation of the rate at all of the concentrations studied corresponded to the initial rate of Pm formation catalyzed by the SK-Pg* complex. This was apparently due to the sequential reactions, the bimolecular reaction conditions of the proteolytic steps, their similar rate constants, differing only by 1.5-fold, and the common cause of inhibition by excess SK. This accounts for the consistent fit of the v_2 dependence for [Lys]Pg activation on SK concentration (Fig. 3A). Simulation of progress curves for this experiment showed that the lower value of K_A = 2 nM fit the data better at [SK]_0 < [Pg]_0, whereas a K_A of ~10 nM produced a better fit to the curves obtained at [SK]_0 > [Pg]_0. This explained the bias in the analysis of the v_2 data and the 5-fold lower apparent K_A obtained from this analysis compared with the analysis of the v_3 dependence on SK concentration.

A similar analysis of the SK dependence of [Glu]Pg activation showed less complex behavior (results not shown). In the [Glu]Pg reactions only 6–9% of Pg was consumed, satisfying the initial rate assumption. The kinetic parameters determined for [Glu]Pg activation by SK-Pm in reactions containing excess

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**Table I**

| Pg species | K_D (nM) | K_A (nM) | k_pg | k_pm | k_S (μM⁻¹ s⁻¹) | k_o (μM⁻¹) | k_pm (μM⁻¹ s⁻¹) |
|------------|----------|----------|------|------|---------------|------------|---------------|
| [Glu]Pgguns | 130 ± 76 | 143 ± 12 | 414 ± 56 | 0.04 ± 0.01 | 19 ± 10 | 0.06 ± 0.02 | 0.003 ± 0.006 |
| [Glu]Pggun | 446 ± 50 | 12 ± 3 | 2.3 ± 0.2 | 0.79 ± 0.02 | 0.27 ± 0.06 | 0.31 ± 0.02 | 1.16 ± 0.04 |
| [Lys]Pgguns | 10 ± 3 | 6 ± 2 | 2.2 ± 0.5 | 0.50 ± 0.05 | 0.003 ± 0.006 |
| [Lys]Pggun | 60 nM and 0.31 | 0.04 | 0.05 | 0.1 | 0.04 | 0.05 | 0.1 |

**Legend for Fig. 4.**

**Fig. 5.** Under these reaction conditions, the rate of total Pm formation (Fig. 5B, Pm_T) was quite linear and agreed well with the initial rate calculated for the increase in total PmA concentration (α + β) from Equation 4. Analysis of the reaction products with increasing SK concentration indicated that inhibition of the rate by excess SK was due to depletion of the free Pg by formation of the SK-Pg* complex, with inhibition of the SK-Pm-catalyzed reaction for the same reason. It was apparent from these results that the modest upward curvature in the rate of total Pm generation from [Lys]Pg at substoichiometric levels of SK would not have been detectable in the experimental progress curves. It was also clear that to within a factor of 2-fold, the linear approximation of the rate at all of the concentrations studied corresponded to the initial rate of Pm formation catalyzed by the SK-Pg* complex. This was apparently due to the sequential reactions, the bimolecular reaction conditions of the proteolytic steps, their similar rate constants, differing only by 1.5-fold, and the common cause of inhibition by excess SK. This accounts for the consistent fit of the v_2 dependence for [Lys]Pg activation on SK concentration (Fig. 3A). Simulation of progress curves for this experiment showed that the lower value of K_A = 2 nM fit the data better at [SK]_0 < [Pg]_0, whereas a K_A of ~10 nM produced a better fit to the curves obtained at [SK]_0 > [Pg]_0. This explained the bias in the analysis of the v_2 data and the 5-fold lower apparent K_A obtained from this analysis compared with the analysis of the v_3 dependence on SK concentration.

A similar analysis of the SK dependence of [Glu]Pg activation showed less complex behavior (results not shown). In the [Glu]Pg reactions only 6–9% of Pg was consumed, satisfying the initial rate assumption. The kinetic parameters determined for [Glu]Pg activation by SK-Pm in reactions containing excess
Pm demonstrated a 70-fold higher $K_g$ of 19 ± 10 μM and a 5-fold lower $K_g$, corresponding to an ~380-fold lower bimolecular rate constant of 0.003 ± 0.006 μM⁻¹ s⁻¹ compared with [Lys]Pg as a substrate (Table I). The parameters for [Glu]Pg activation resulted in simulated linear reaction progress curves approximating closely the initial rates of SK-Pg* catalysis of Pg activation with little contribution from the much slower SK-Pm-catalyzed pathway. Values of $K_g$ from previous analyses of $v_1$ (143 ± 12 nm) and $v_2$ (414 ± 56 nm) were in reasonable agreement for [Glu]Pg when fit by the model lacking the SK-Pm-catalyzed reactions (Table I).

A model in which all Pm was generated by intramolecular cleavage of SK-Pg*, as proposed previously (20–22), did not fit the observed $v_2$ data, particularly for [Lys]Pg, because it predicted no inhibition in $v_2$ with increasing SK concentration. Analysis of the data with an expanded version of Scheme 3, including the possibility of intramolecular proteolytic conversion of SK-Pg* to SK-Pm, did not fit the $v_2$ dependence on SK concentration as well and required a 10–20-fold higher rate of intramolecular cleavage to account for the data. Including this first-order generation of SK-Pm resulted in higher rates of Pm formation in excess SK because the generation of SK-Pm was not inhibited by depletion of free Pg by SK binding. These results ruled out intramolecular cleavage as a significant reaction in activation of [Lys]Pg by SK.

DISCUSSION

The results of quantitative equilibrium binding and kinetic studies presented here and in the preceding paper (16) support a unified hypothesis for the mechanism of SK-induced conformational and proteolytic activation of Pg is illustrated. Free Pg is represented as an extended form with the N-terminal peptide (squiggle) attached to the five kringle domains and the proteinase domain. This has been done to simplify the scheme and is not meant to be restricted to this form of [Glu]Pg but to represent both conformational forms of [Glu]Pg and [Lys]Pg. Interactions of SK with Pg and Pm involving lysine-binding sites in kringles 4 and 5 are also not represented to simplify the scheme. The reaction proceeds through the indicated complexes. The upper catalytic cycle is initiated by Pg-SK binding and conformational activation in formation of Pg*SK, which binds Pg in the substrate mode, forming the ternary Michaelis complex, Pg*SK-Pg, from which initial Pm is produced. Pm generated by Pg*SK binds tightly to free SK in the catalytic mode, forming Pm-SK in competition with Pg*SK. The tightly bound Pm-SK complex initiates the lower catalytic cycle, binding free Pg in the substrate mode to form Pm-SK-Pg, from which free Pm is generated.

The mechanism explains the inhibition of Pm formation by high SK concentration and the larger inhibition seen for [Lys]Pg compared with [Glu]Pg. The first proteolytic cycle is inhibited by high SK concentration because of the dual role of Pg as the catalytic component of Pg*SK and as substrate for this complex. At high SK concentration, proteolytic Pg activation is inhibited because the free Pg concentration is depleted by formation of the Pg*SK complex. As predicted by this model for the kinetics of [Lys]Pg activation, for which the affinity for
SK is relatively high, the maximum rate occurs at \(-2\) mol Pg/mol SK, reflecting the concentrations required for optimum formation of the productive Pg*-SK-Pg ternary Michaelis complex (Fig. 6). For [Glu]Pg, the reaction is also inhibited by high SK concentration but inhibition is weaker because of the lower affinity of SK for [Glu]Pg. The results show that the rate of Pm formation from [Lys]Pg approaches zero at high SK concentrations. A mechanism involving generation of Pm by intramolecular cleavage within the Pg*-SK complex proposed previously (20–22) does not fit the data because the first-order generation of Pm would not be inhibited by SK. A mixed intramolecular-intermolecular mechanism also predicts incomplete inhibition by SK. These results support the conclusion that Pm is formed initially by intermolecular cleavage of free Pg by Pg*-SK. The essentially complete inhibition of Pm formation observed for [Lys]Pg also indicates that proteolytic formation of Pm by Pg*-SK acting on other Pg*-SK complexes is at most a very slow process. The kinetics of Pm formation for both [Glu]Pg and [Lys]Pg as a function of SK and Pg concentration were well described by the intermolecular model, which predicts complete inhibition at saturating SK concentration.

An initial assumption was necessary for analysis of the rates of Pm generation measured by continuous monitoring of chromogenic substrate hydrolysis. The measured rates were inhibited hyperbolically by d-Val-Leu-Lys-pNA with an apparent inhibition constant of \(330 \pm 80\ \mu\text{M}\). The inhibition constant was essentially the same as \(K_a\) for the Pm-SK complex (Scheme 2) (15), suggesting that inhibition of the contribution of Pm-SK-catalyzed Pm formation to the measured rate of total Pm generation could be responsible. It is also possible, however, that negative linkage between chromogenic substrate binding to Pg*-SK and the affinity of SK binding and conformational activation (\(K_a\)) could account for the observed inhibition (16). Simulation of the reactions showed that the rate of Pm generation was very sensitive to small changes in \(K_a\). Because this issue could not be resolved unambiguously, experiments were performed at a constant substrate concentration, and the measured \(v_2\) rates were corrected for 1.4-fold inhibition at this concentration. The appropriateness of this correction was supported by the normalization of the continuously and discontinuously measured rates with the above inhibition constant, where the latter were not subject to inhibition by substrate. Because this effect was small and the correction was constant for all of the rates, it does not change the interpretation of the large differences in the kinetic constants demonstrated or affect the main conclusions regarding the mechanism.

Pm generated and released in the first catalytic cycle (Fig. 6) binds free SK in the catalytic mode with extremely high affinity. Results of equilibrium binding studies for the native proteins yielded dissociation constants of \(130 \pm 76\ \text{nM}\) for [Glu]Pg, \(10 \pm 3\ \text{nM}\) for [Lys]Pg, and \(0.012 \pm 0.004\ \text{nM}\) for Pm (15, 16), representing 800- and 11,000-fold higher affinity of SK for Pm compared with [Lys]Pg and [Glu]Pg, respectively. As expected from these large differences in affinity, and as shown in the companion paper (16), Pm readily displaces Pg from the Pg*-SK complex. This indicates that the initial Pm generated will sequester SK in the tightly bound Pm-SK complex.

The Pm-SK complex initiates the lower catalytic cycle in Fig. 6, which consists of exosite-mediated Pg binding as a substrate and its proteolytic activation to generate additional Pm (15). Characterization of these reactions for [Glu]Pg and [Lys]Pg as substrates of Pm-SK demonstrated an \(-380\)-fold lower bimolecular rate constant for [Glu]Pg, due primarily to a 70-fold higher \(k_{\text{cat}}\) and to a lesser extent 5-fold lower \(k_{\text{off}}\). Although the low specificity of the Pm-SK complex for [Glu]Pg compared with [Lys]Pg appears to be due primarily to lower affinity binding of [Glu]Pg as a substrate, whether this reflects a difference in lysine-binding site interactions, which seems likely, has not yet been determined.

Despite the sequential nature of the Pg*-SK and Pm-SK-catalyzed reactions, the experimentally determined progress curves of total Pm formation under our conditions did not reveal the nonlinearity possible for these reactions. Moreover, the dependence of \(v_2\) on Pg and SK concentration was reproduced by a model including only the Pg*-SK catalytic cycle and excluding the subsequent conversion of Pg to Pm by Pm-SK. To investigate the reasons for this, the full reaction scheme (Scheme 3) was simulated with the simplifying assumptions that both proteolytic reactions were bimolecular over the range of Pg concentration studied. Applying this assumption was justified for Pg*-SK-catalyzed Pg cleavage from the analysis of the rates of conformational activation (\(v_1\)) as a function of SK and Pg concentration (16). The Pm-SK-catalyzed reactions could also be treated as bimolecular, based on the Pg concentrations being much lower than the determined \(K_a\). With these assumptions and the determined kinetic parameters, simulation of the time courses of the reaction species demonstrated the behavior expected for the sequential reactions. For [Lys]Pg activation, however, the progress curves of total Pm formation curved upward only modestly at substoichiometric (\([\text{SK}]_0 < [\text{Pg}]_0\)) levels of SK and were quite linear at \([\text{SK}]_0 > [\text{Pg}]_0\) concentrations. Analysis of the simulated progress curves demonstrated that at \([\text{SK}]_0 < [\text{Pg}]_0\) the measured rates were overestimated by a maximum of 2-fold compared with the rates calculated for the model including only the Pg*-SK-catalyzed reaction. This simplified model fit the \(v_2\) data as a function of SK and [Lys]Pg concentration very well, but with an \(-5\)-fold higher apparent affinity for the conformational activation equilibrium constant (\(K_a\)) of 2.3 \(\pm 0.2\ \text{mM}\) compared with 12 \(\pm 3\ \text{mM}\) determined from analysis of \(v_1\). The bias in the linear fits of the progress curves at \([\text{SK}]_0 < [\text{Pg}]_0\) accounted for this effect and indicated that the true dissociation constant was more likely 5–10 mM, in better agreement with the analysis of \(v_1\) and results of the equilibrium binding studies. The bimolecular rate constants for [Lys]Pg activation by Pg*-SK and Pm-SK were similar, differing by only 1.5-fold. The combination of first-order conditions for the proteolytic reactions with similar rate constants and the common mechanism of inhibition of the rate by depletion of free Pg resulted in the near-linear progress curves and good fit by the mechanism including only Pg*-SK-catalyzed Pg activation. The situation for [Glu]Pg activation was less complicated, and the parameters determined from analysis of \(v_1\) and \(v_2\) by fitting of the model containing only the Pg*-SK reaction were in good agreement. This was due to the lower affinity of SK for binding and conformational activation of [Glu]Pg and the \(-13\)-fold lower bimolecular rate constant for catalysis by Pm-SK compared with Pg*-SK, resulting in a lesser contribution of the Pm-SK-catalyzed cycle to total Pm formation.

Comparison of the bimolecular rate constants for Pg*-SK and Pm-SK-catalyzed [Glu]Pg and [Lys]Pg activation demonstrated an \(-20\)-fold slower rate of [Glu]Pg activation by Pg*-SK, whereas Pm-SK catalyzed activation of [Glu]Pg with an \(-380\)-fold slower rate. These results and the similar rate constants obtained for [Lys]Pg activation demonstrated that Pg*-SK and Pm-SK have different specificities for [Glu]Pg and similar specificities for [Lys]Pg as substrates. The source of the more efficient catalysis of [Glu]Pg activation by Pg*-SK is not known but may reflect changes in the catalytic site accompanying SK binding that result in the lower tripeptide chromogenic substrate specificity demonstrated for Pg*-SK compared with Pm-SK (16). It is also possible that exosite-mediated
tightly bound Pm-SK complex generated then propagates full conversion of the remaining Pg to Pm. Rapid and reversible binding and conformational activation of Pg by SK and an initial round of intermolecular Pg activation catalyzed by Pg-SK is thus a self-limiting process that produces one SK equivalent of the irreversibly activated Pm-SK complex, which becomes the sole catalyst in the full conversion of Pg to Pm.

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