Resolution of Conformational Activation in the Kinetic Mechanism of Plasminogen Activation by Streptokinase*

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Streptokinase (SK) activates plasminogen (Pg) by specific binding and nonproteolytic expression of the Pg catalytic site, initiating Pg proteolysis to form the fibrinolytic proteinase, plasmin (Pm). The SK-induced conformational activation mechanism was investigated in quantitative kinetic and equilibrium binding studies. Progress curves of Pg activation by SK monitored by chromogenic substrate hydrolysis were parabolic, with initial rates (v1) that indicated no transient species and subsequent rate increases (v2). The v1 dependence on SK concentration for [Glu]Pg and [Lys]Pg was hyperbolic with dissociation constants corresponding to those determined in fluorescence-based binding studies for the native Pg species, identifying v1 as rapid SK binding and conformational activation. Comparison of [Glu]Pg and [Lys]Pg activation showed an ~12-fold higher affinity of SK for [Lys]Pg that was lysine-binding site dependent and no such dependence for [Glu]Pg. Stopped-flow kinetics of SK binding to fluorescently labeled Pg demonstrated at least two fast steps in the conformational activation pathway. Characterization of the specificity of the conformationally activated SK-[Lys]Pg* complex for tripeptide-p-nitroanilide substrates demonstrated 5–18- and 10–130-fold reduced specificity (kcat/Km) compared with SK-Pm and Pm, respectively, with differences in Km and kcat dependent on the P1 residue. The results support a kinetic mechanism in which SK binding and reversible conformational activation occur in a rapid equilibrium, multistep process.

Streptokinase (SK)* is used clinically as a thrombolytic drug to activate plasminogen (Pg) to plasmin (Pm), the serine proteinase responsible for dissolution of fibrin clots (1). Native [Glu]Pg is a multidomain zymogen consisting of a 77-residue N-terminal peptide, five kringle domains, and a serine proteinase catalytic domain that is activated by cleavage of Arg561–Val562 (2, 3). [Glu]Pg is in a compact conformation, maintained by intramolecular interactions between the N-terminal peptide and lysine-binding sites in kringles 4 and 5 (4–6). Release of the N-terminal peptide by Pm cleavage generates [Lys]Pg, which adopts an extended conformation and is cleaved by plasminogen activators at a faster rate (5, 7–13). SK possesses no intrinsic catalytic activity but interacts with Pg and Pm, converting both thezymogen and active proteinase into specific proteolytic Pg activators (14–19). SK binding to Pg results in conformational expression of an active catalytic site on the zymogen without the usual strict requirement for peptide bond cleavage (14, 16, 17). Pm is generated subsequently by proteolytic cleavage of Arg561–Val562, and the SK-Pm complex propagates Pg activation through expression of a substrate recognition exosite (20, 21).

It is well established that both conformational and proteolytic activation contribute to SK-induced Pg activation, but there are a number of unresolved questions concerning the mechanism of conformational activation and its coupling to subsequent proteolytic Pm formation. Early studies (14, 16, 17) demonstrated that interaction of SK with Pg produced the activated Pg catalytic site in the SK-Pg* complex. Subsequent kinetic studies indicated that Pg activation involved an initially formed SK-Pg* activation complex and an isomerized form of the complex (22, 23). The isomerization was time-, chloride ion-, and fibrinogen-dependent, and the active complexes interconverted slowly under certain experimental conditions. In other studies, a species of Pg isolated from a mixture of SK and Pg was reported to be an active “virgin enzyme” form of free Pg*, suggesting irreversible conformational activation (24). The relationship between SK-Pg binding and conformational activation and its reversibility have not been clearly established.

Previous studies of the mechanism of Pg activation by SK have not taken into account the binding affinities of SK for Pg and Pm species, primarily because consistent and reliable equilibrium binding constants have not been available. A number of studies have examined the binding interactions by various experimental approaches but have resulted in a very broad range of dissociation constants, varying from 28 pM to 220 nM for [Glu]Pg (25–32), for example, where the higher affinities may result from Pg generation in SK/Pg mixtures. Our equilibrium binding studies have employed active site-labeled fluorescent Pg and Pm analogs to quantitate SK binding in the absence of proteolytic reactions (21, 28, 33, 34). The results for the active site-labeled proteins support a relatively low affinity of SK for [Glu]Pg, a 13–16-fold enhanced affinity for [Lys]Pg due to the expression of lysine-binding site interactions and a 3100–3500-fold further enhancement accompanying activation of the Pg catalytic domain. These changes in affinity account for a remarkable, overall ~50,000-fold higher affinity of SK.
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for the reaction product, [Lys]Pm, compared with the initial substrate, [Glu]Pg (21, 33).

Lysine-binding sites on the kringle domains of Pg and Pm participate in SK-induced Pg activation by mechanisms that are only partly understood. Conversion of Pg to Pm is inhibited by the lysine analog, 6-aminohexanoic acid (6-AHA) (35, 36). In our studies, 6-AHA reduced binding affinity of SK for [Lys]Pg and [Lys]Pm only partially but did not affect the affinity of SK for [Glu]Pg (21, 28, 33), whereas others (36) have reported weakening of [Glu]Pg binding by 6-AHA. The role of lysine-binding site interactions in modulating conformational activation of [Glu]Pg and [Lys]Pg is not clear.

The goal of the present studies was to apply, for the first time, the combination of quantitative equilibrium binding and kinetic analysis required to define the mechanism of SK-induced Pg activation. Resolution of the conformational activation steps in the kinetic mechanism is characterized in the present paper, and coupling of the conformational and proteolytic activation pathway is reported in the companion paper (37). The studies presented here address the relationship between SK-Pg binding and conformational activation, the nature of Pg*, the unknown role of differences in affinities of SK for [Glu]Pg and [Lys]Pg, and the roles of lysine-binding sites. The results demonstrate that Pg activation can be kinetically resolved into consecutive conformational and proteolytic activation steps at pH 7.4, I 0.15 M, and 25 °C. Conformational activation is induced by rapid equilibrium binding of SK to Pg and an accompanying reversible conformational change to form SK-Pg*. The mechanism of SK binding to fluorescence-labeled Pg involves at least two steps that can be resolved on the stopped-flow time scale. Kinetic separation of conformational and proteolytic activation allowed the substrate specificity of the transiently formed SK-Pg* complex to be characterized. The SK-Pg* complex exhibited significant decreases in substrate specificity for tripeptide p-nitroanilide substrates compared with that demonstrated previously for SK-Pm or Pm (21), indicating a unique active site specificity for Pg* in the SK-Pg* complex. The combined equilibrium binding and kinetic data under our experimental conditions did not reveal slowly formed, functionally distinguishable activator complexes and did not support formation of a virgin enzyme. These studies provide the first quantitative equilibrium binding and kinetic analysis of conformational Pg activation by SK, and demonstrate the critical role of differences in SK affinity for [Glu]Pg and [Lys]Pg and the differential roles of lysine-binding site interactions in regulating the activation mechanism.

EXPERIMENTAL PROCEDURES

Protein Purification and Characterization—[Glu]Pg was purified from human plasma and separated into carbohydrate variants Pg1 and Pg2 by minor modifications of published procedures (38, 39). Pg was prepared by activation of [Glu]Pg as described previously (28, 40).

Kinetic Model of Plasminogen Activation by Streptokinase—The observed dependence of ν on SK concentration can be described by the mechanism shown in Scheme 1.

The analysis gave the initial rate of substrate hydrolysis at the beginning of the reaction ν(0) and the rate of increase in activity with time (ν(t)). Linear transformation of the continuous assay time courses was accomplished by dividing ΔA 405 nm by t (52).

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In this model, SK binds to Pg with the dissociation constant $K_s$ to form a reversible, conformationally activated SK-Pg* complex, which can bind chromogenic substrate (S) with Michaelis constant $K_m$ and generate Pm with the catalytic rate constant $k_c$. This study characterizes Pg binding to SK, resolves the processes involved in conformational activation of Pg resulting in formation of the activated SK-Pg* complex, and quantifies the kinetic parameter $K_s$ for Pg binding. The companion paper (37) describes the close proteolytic mechanism of Pm generation by the activated SK-Pg* complex and formation of the SK-Pm complex that catalyzes Pg activation to Pm.

The observed progress of activity formation is given by Equation 1, where $v_1$ and $v_2$ represent the rate of chromogenic substrate turnover by SK-Pg* and the rate of generation of Pm, respectively. From the Michaelis-Menten equation and the equilibrium binding expression for SK binding to Pg, $v_1$ for the model in Scheme 1 is given by Equation 2, where $[S]_0$ is the total substrate concentration.

$$v_1 = \frac{k_1[Pg][Pg^*][S][S]}{K_a + [S]}$$  

Solving the SK mass balance equation for $[SK]_{free}$ in terms of $[Pg]_{free}$ gave Equation 3, where $[SK]_0$ is the total SK concentration,

$$[SK]_{free} = \frac{[SK]_0}{1 + \frac{[S]}{K_a}}$$  

and substitution of Equation 3 into Equation 2 gave Equation 4.

$$v_1 = \frac{k_1[Pg][SK][S]}{K_a + [S]}$$

Solving the mass conservation equation for $[Pg]_{free}$ gave the cubic Equation 5 as an exact solution in terms of the total concentrations, dissociation, and kinetic constants.

$$D_1([Pg]_{free})^3 + D_2([Pg]_{free})^2 + D_3([Pg]_{free}) + D_4 = 0$$

The dependence of the rates of Pg activation on SK Concentration—The dependence of Pg activation on SK concentration was investigated in continuous activity assays in which Pg was activated with SK in the presence of 200 μM VLK-pNA, and the reaction was monitored by the increase in absorbance at 405 nm with time. Progress curves at low SK concentrations were parabolic, becoming increasingly linear at higher SK concentrations (Fig. 1A). No lags were observed in the absorbance change over time, linear transformations of the progress curves, indicating rapid formation of active species. Analysis of the progress curves in a model-independent manner by fitting a second order polynomial (Equation 1) resulted in the rates $v_1$ and $v_2$, which are described by the quadratic binding equation with an apparent dissociation constant of 10–5 nm and a maximum rate of 0.020 μM s⁻¹. By contrast, the dependence of $v_2$ on SK was very unusual, increasing sharply to a maximum of 2 × 10⁻³ μM s⁻¹ at an SK concentration approximately equal to one-half that of Pg and decreasing to essentially zero at high SK concentrations.

Dependence of the Rates of Pg Activation on SK Concentration—The dependence of Pg activation on SK concentration is shown (Fig. 1B). The dependence of $v_1$ on SK was hyperbolic and well described by the quadratic binding equation with an apparent dissociation constant of 10 ± 5 nm and a maximum rate of 0.020 ± 0.001 μM s⁻¹. By contrast, the dependence of $v_2$ on SK was very unusual, increasing sharply to a maximum of 2 × 10⁻³ μM s⁻¹ at an SK concentration approximately equal to one-half that of Pg and decreasing to essentially zero at high SK concentrations.

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Fig. 1. Activation of [Lys]Pg by SK. A, progress curves of the absorbance change at 405 nm (A vs. m) are shown for reactions of 10 nM [Lys]Pg, with 1 (curve a), 2 (curve b), 3 (curve c), 4 (curve d), 200 (curve e), and 500 nM (curve f) SK in the presence of 200 μM VLK-pNA. B, v1 (●) and v2 (○) are from these and other data obtained as described under "Experimental Procedures" plotted versus the total SK concentration ([SK]0). Solid lines represent the least squares fit of the vi data by the quadratic binding equation, whereas a simple connecting line is shown for the v2 data. Assays were performed and analyzed as described under "Experimental Procedures."

Fig. 2. Kinetics of [Lys]Pg and [Glu]Pg activation by SK. The dependence of v1 obtained from analysis of reactions of 2 (●), 5 (○), 10 (A), 15 (△), and 20 nM (■) [Lys]Pg (A), and from reactions of 2 (●), 10 (○), 15 (A), and 20 nM (△) [Glu]Pg with SK in the presence of 200 μM VLK-pNA (B) are shown as a function of total SK concentration ([SK]0). Lines represent a global fit to the data by Equations 11 and 12. Assays were performed and analyzed as described under "Experimental Procedures."

and that the proteolytic reaction could be treated as bimolecular. The fitted v1 dependence yielded k1/Km of 12 ± 1 nM s⁻¹ and K1 12 ± 3 nM for [Lys]Pg1 activation by SK, k2/Km of 11 ± 1 nM s⁻¹, and K2 143 ± 12 nM for [Glu]Pg activation (Table I).

Binding of SK to Native and Fluorescently Labeled [Lys]Pg and [Glu]Pg—To determine whether the observed increase in v1 with SK represented rapid binding and concomitant formation of the SK-Pg* complex, the dissociation constants for SK binding to native [Lys]Pg and [Glu]Pg were measured and compared with the kinetically determined parameters. Fig. 3A shows the results of a competitive fluorescence titration of 15 nM fluorescein-labeled [Lys]Pg1, (5-F)FPR[Lys]Pg1 and 100 nM SK with native [Lys]Pg1. In these experiments, the fluorescence changes accompanying rapid equilibration of the competitive binding equilibria on addition of SK to mixtures of labeled and unlabeled Pg were kinetically resolved from slower changes accompanying proteolytic Pg formation (28). Fitting of the competitive binding model to these data gave a dissociation constant of 42 ± 5 nM for SK binding to labeled [Lys]Pg1, a dissociation constant of 10 ± 3 nM for SK binding to native [Lys]Pg, and a maximum fluorescence change of −29 ± 1%. Analysis of similar titrations of (5-F)FPR[Glu]Pg1 and SK with [Glu]Pg1 (Fig. 3B) yielded dissociation constants of 686 ± 113 and 130 ± 76 nM for SK binding to labeled and native [Glu]Pg1, respectively, and the same maximum fluorescence change. The dissociation constant for SK binding to native [Glu]Pg was consistent with the value of 90 ± 100 nM determined previously by kinetically resolved competitive fluorescence titration with 2-anilinonaphthalene-6-sulfonic acid-labeled [Glu]Pg1 (28). The close correspondence between the dissociation constants for both native [Glu]Pg and [Lys]Pg and the constants determined from the activation kinetics showed that v1 represented the rate of hydrolysis of chromogenic substrate by SK-Pg* and that the increase in v1 with increasing SK concentration represented rapidly established binding of SK to Pg to form the conformationally activated SK-Pg* complex.

Effect of 6-AHA on SK Binding and Conformational Activation—The involvement of Pg lysine-binding sites in SK-induced activation was investigated in binding and kinetic studies of the effect of 6-AHA. Continuous assays of 10 nM [Lys]Pg1 and [Glu]Pg1 activation as a function of SK concentration were performed in the absence and presence of near-saturating 10 mM 6-AHA (Fig. 4). Analysis of titrations of v1 with SK using Equations 11 and 12 gave an apparent dissociation constant of 50 ± 5 nM for [Lys]Pg1, which was a 4-fold decrease in affinity from that determined in the absence of 6-AHA (Table I). The increase in v1 with SK concentration for [Glu]Pg1, which gave similar apparent dissociation constants of 143 ± 12 and 65 ± 9 nM in the absence and presence of 6-AHA, respectively, indicated no significant differences in affinity of SK for [Lys]Pg and [Glu]Pg in 6-AHA. The effect of 6-AHA on the maximum activity for [Glu]Pg and [Lys]Pg was a modest decrease, most likely the result of weak inhibition of chromogenic substrate hydrolysis by 6-AHA (53), resulting in subtle differences in maximum activities of 0.017 ± 0.001 μM s⁻¹ for [Lys]Pg and 0.021 ± 0.002 μM s⁻¹ for [Glu]Pg in 6-AHA (Fig. 4 and Table I).

Competitive fluorescence binding studies for native Pg in the presence of 10 mM 6-AHA showed that SK bound native [Lys]Pg1 12-fold more weakly, with a dissociation constant of 115 ± 32 nM (Fig. 3A) and 530 ± 45 nM for SK binding to labeled Pg. In contrast to the results for [Lys]Pg, competitive fluorescence titrations of [Glu]Pg showed no significant effect of 6-AHA on the affinity for SK. SK bound to native [Glu]Pg1 with a dissociation constant of 114 ± 32 nM and to labeled Pg with a dissociation constant of 737 ± 49 nM (Fig. 3B and Table
Dissociation constants ($K_D$) are listed for SK binding to the indicated labeled and native Pg species obtained from direct and competitive fluorescence titrations in the absence and presence of 10 mM 6-AHA. Also listed are kinetically determined dissociation constants ($K_A$ ($v_1$)) for native SK-Pg interactions and the specificity constants ($k_{cat}/K_m$) for chromogenic substrate hydrolysis by the SK-saturated complexes in the absence and presence of 10 mM 6-AHA. Kinetic and binding studies were performed, and the data were analyzed as described under “Experimental Procedures.”

![Table I](image)

Table I

Equilibrium binding and kinetic constants for SK-plasminogen interactions

| Pg species | $K_D$ (nM) | $K_A$ ($v_1$) | $k_{cat}/K_m$ (μM$^{-1}$ s$^{-1}$ $\times 10^{-3}$) | $K_D$ (nM) | $K_A$ ($v_1$) | $k_{cat}/K_m$ (μM$^{-1}$ s$^{-1}$ $\times 10^{-3}$) |
|------------|------------|--------------|---------------------------------|------------|--------------|---------------------------------|
| [5-F]FFR(Glu)Pg | 686 ± 113 | 11 ± 1 | 15 ± 1 | 737 ± 49 | 11 ± 1 | 530 ± 45 |
| [Glu]Pg | 130 ± 76 | 143 ± 12 | 15 ± 1 | 114 ± 32 | 65 ± 9 | 115 ± 32 |
| [Glu]Pg2 | 42 ± 5 | 12 ± 3 | 15 ± 1 | 9 ± 1 | 15 ± 1 | 50 ± 5 |
| [Lys]Pg | 10 ± 3 | 6 ± 2 | 15 ± 1 | 42 ± 5 | 12 ± 3 | 50 ± 5 |
| [Lys]Pg2 | 10 ± 1 | 3 | 15 ± 1 | 42 ± 5 | 12 ± 3 | 50 ± 5 |

Fig. 3. Competitive binding of native [Lys]Pg and [Glu]Pg with fluorescein-labeled Pg to SK. A, the change in fluorescence ($\Delta F/F_o$) of 15 nM [5-F]FR(Lys)Pg and 100 nM SK versus total native [Lys]Pg concentration ([Pg]o) is shown in the absence (○) and presence (●) of 10 mM 6-AHA. B, the change in fluorescence ($\Delta F/F_o$) of 15 nM [5-F]FR(Glu)Pg and 500 nM SK is plotted as a function of [Glu]Pg concentration ([Pg]o) in the absence (○) and presence (●) of 10 mM 6-AHA. Lines represent nonlinear least squares fits of each data set by the cubic competitive binding equation for native and labeled Pg binding to SK. Fluorescence titrations were performed and analyzed as described under “Experimental Procedures.”

I. The decreased affinity of SK for native [Lys]Pg and not [Glu]Pg was comparable with that reported previously for the fluorescent labeled Pg species (28, 33). These results demonstrated quantitative correspondence between the kinetically determined dissociation constants for conformational activation and the dissociation constants determined in the competitive equilibrium binding studies in the absence of lysine-binding site interactions.

The effect of the active site label on the affinity of SK for [Lys]Pg and [Glu]Pg was indistinguishable for [Lys]Pg1 and [Glu]Pg1, which exhibited 4.2- and 5.3-fold weaker binding to SK compared with the native proteins, respectively, in the absence of 6-AHA, and 4.6- and 6.5-fold weaker binding, respectively, in 10 mM 6-AHA (Table I). This was consistent with the 6.5-fold decrease reported previously (28) for [Glu]Pg labeled with 2-(4′-iodoacetamido)anilino)naphthalene-6-sulfonic acid, thus indicating a consistent effect of occupation of the active site by the probe and linking peptide on SK affinity and conformational activation.

The results of the native protein competitive titrations established that the labeled proteins bound SK with ~5-fold lower affinity, but importantly, these effects were similar for [Lys]Pg and [Glu]Pg. The effect of 6-AHA on the interaction of SK for the native and labeled proteins was not altered by this ~5-fold difference in affinity.

The role of the compact to extended conformational change in [Glu]Pg activation was investigated by comparison of activation of [Glu]Pg with SK in buffer containing 125 mM Cl$^-$, which stabilizes the compact conformation of [Glu]Pg with buffer in which Cl$^-$ had been replaced with acetate, which allows [Glu]Pg to adopt the extended conformation (4, 11, 12, 54). Dependence of $v_1$ on SK at 1 and 10 nM [Glu]Pg2 indicated a $K_A$ ~2 nM (results not shown), consistent with the previously determined $K_A$ of 11 nM for binding of fluorescein-labeled [Glu]Pg (33) and an ~5.5-fold lower affinity for the labeledzymogen. As observed by equilibrium binding and by kinetics, SK preferentially bound and conformationally activated the extended conformation of [Glu]Pg.

Pre steady-state Kinetics of Conformational Activation—Rapid-reaction kinetic studies were performed to investigate SK-Pg interactions in the fast conformational activation process forming SK-Pg*. As shown in Fig. 5 for [5-F]FFR(Lys)Pg, stopped-flow fluorescence time traces of SK binding to [5-F]FFR-[Glu]Pg2 and [5-F]FFR[Lys]Pg were biexponential, with fast phases characterized by pseudo-first-order rate constants of 12 ± 2 and 22 ± 4 s$^{-1}$, respectively, and slower phases with indistinguishable rate constants of 0.3–0.5 s$^{-1}$ for both interactions.

In the presence of 6-AHA, the first exponential phase of the SK interaction with [5-F]FFR[Glu]Pg2 was unchanged, whereas with [5-F]FFR[Lys]Pg, the rate constant decreased by 50% to the value observed for [5-F]FFR[Glu]Pg2 (Fig. 5). The
buffer containing 50 mM 6-AHA for 2 min resulted in formation of SK-Pg* complex formed by reaction of 10 nM [Lys]Pg, with 50 nM SK are plotted as a function of time, in the absence (curve 1) and presence (curve 2) of 50 mM 6-AHA. The lines represent nonlinear least squares fits of each data set by a biexponential equation. Stopped-flow data were captured and analyzed as described under “Experimental Procedures.”

slow phases were unaffected by 6-AHA. The results demonstrated that binding of SK and conformational activation involves at least two fast steps, one of which is dependent on lysine-binding site interactions.

**Dissociation of the SK-Pg* Complex with Active Site-blocked Pm**—To determine whether conformational activation of Pg by SK was due to a reversible conformational change or to irreversible generation of conformationally activated Pg* virgin enzyme via the plasminogen activator complex, SK-Pg* was formed rapidly and then incubated with Pm blocked with d-Phe-Phe-Arg-CH2Cl (FFR-Pm), which binds very tightly to SK (21, 33). Incubation of 10 nM [Lys]Pg1 with 50 nM SK in buffer containing 50 mM 6-AHA for 2 min resulted in formation of the activated SK-Pg* complex, which could be quantitated from the initial rates of chromogenic substrate hydrolysis. Subsequent addition of FFR-Pm resulted in decreased activity of the complex, consistent with rapid competitive binding of SK to [Lys]Pg and FFR-Pm and dissociation of SK-Pg* to free SK and inactive zymogen (Fig. 6). The reversibility of complex formation was further demonstrated in assays in which SK was added to a pre-quenched mixture of FFR-Pm, 10 nM [Lys]Pg, and substrate, with indistinguishable results (Fig. 6). Analysis of the data with the cubic binding equation for competitive binding of [Lys]Pg and FFR-Pm to SK gave a dissociation constant of 23 ± 21 nM for SK binding to native [Lys]Pg and a maximum activity of 0.013 ± 0.005 μM s⁻¹ for the SK-Pg* complex. These parameters were in agreement with previously determined values of 50 ± 5 nM and 0.017 ± 0.001 μM s⁻¹ (Fig. 4 and Table I). These results demonstrated rapid reversibility of SK-Pg* complex formation and provided no evidence for formation of an irreversibly activated virgin enzyme species.

**Kinetic Parameters of Tripeptide-p-Nitroanilide Substrate Hydrolysis by the SK-Pg* Complex**—Resolution of conformational activation from proteolytic reactions in Pg activation allowed for the first time a detailed investigation of the kinetic properties of the transiently formed SK-Pg* complex. Kinetic parameters were determined from v₁ rates of [Lys]Pg₂ activation in the presence of saturating SK for hydrolysis of an array of 1 dipeptide and 11 tripeptide substrates, including a peptide with the sequence of the plasminogen activation cleavage site (PGR-pNA) (Table II). The previously reported parameters for hydrolysis of the same substrates by the SK-Pm complex are shown for comparison (21). The results demonstrated reduced kₐ/Kₘ values representing losses of 5–18 and 10–130-fold in substrate specificity of SK-Pg* when compared with SK-Pm (Table II) or free Pm, respectively (21). Differences in kinetic parameters for hydrolysis by SK-Pm and SK-Pg* were dependent on the identity of the substrate P1 residue. The driving force behind the decrease in specificity of SK-Pg* compared with SK-Pm for substrates with Lys in the P1 position was a 650–1500% increase in Kₘ accompanied by modest effects of –12 to 60% on kₐ (Fig. 7). In contrast, substrates with Arg at P1 exhibited 43–97% decreases in kₐ and small effects on Kₘ ranging from a 55% decrease to a 400% increase, with the exception of pyro-EPR-pNA, which exhibited an 1180% increase (Table II and Fig. 7). These results demonstrated significant loss of substrate specificity between SK-Pm and SK-Pg*, and suggested that these changes were dependent on differences in the interactions of the S1 specificity subsite of the enzymes with the P1 residue of the substrates. The decreased specificity of SK-Pg* and SK-Pm for PGR-pNA relative to Pm (21) demonstrated that changes in the S1-S3 specificity subsites play little or no role in the specificity for conversion of Pm or Pg into proteolytic Pg activators.

**DISCUSSION**

Resolution of conformational activation from subsequent proteolytic reactions in the kinetic mechanism of SK-induced Pg activation, in conjunction with analysis of SK binding to native [Glu]Pg and [Lys]Pg, allowed the quantitative relationship between SK-Pg binding and conformational activation to be established for the first time. Conformational activation of Pg was readily reversible, and the results provided no evidence for irreversible formation of the free, activated virgin form of Pg* postulated previously (24). SK binding to Pg and conformational expression of the Pg catalytic site in the SK-Pg* complex occurred rapidly and without rate-controlling intermediates on the seconds time scale. The slow (minutes) isomerization of the SK-Pg* complex reported in previous studies (22, 23) with [Glu]Pg at low temperature and/or low chloride concentrations was not observed in our studies. This does not mean that this conformational change does not occur but likely represents differences in the experimental conditions, where this event apparently occurs rapidly under our conditions.

Although several previous studies have sought to quantitate binding of SK to Pg/Pm species, the results have not yielded consistent affinities, and in no studies has equilibrium binding of SK been related directly to nonproteolytic Pg activation. The approach based on active site-labeled fluorescent Pg analogs enabled quantitation of the dissociation constants for native [Glu]Pg and [Lys]Pg by resolving the rapid competitive binding equilibria measured by fluorescence from slower proteolytic reactions. The results demonstrate the quantitative corre-
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The specificity constants for hydrolysis of the indicated trip -

TABLE II

Effect of SK binding to [Lys]PG* and Pm* on hydrolysis of tripeptide-pNA substrates

Kinetic parameters were determined by nonlinear least squares fitting of the Michaelis-Menten equation to $v_i$ rates versus substrate concentration data as described under “Experimental Procedures.” NA represents a detectable activity. Results for SK-Pm* were taken from Ref. 21. CBO, carbobenzoxy.

| Substrate        | SKPm* | SK[Lys]PG* |
|------------------|-------|-----------|
|                  | $k_i$ | $K_m$     | $k_i/K_m$ | $k_i$ | $K_m$ | $k_i/K_m$ |
|                  | $s^{-1}$ | $\mu M$ | $s^{-1}$ | $\mu M$ | $s^{-1}$ | $\mu M$ | $s^{-1}$ | $\mu M$ | $s^{-1}$ | $\mu M$ | $s^{-1}$ | $\mu M$ | $s^{-1}$ | $\mu M$ |
| n-Val-Kp-pNA     | 34 ± 2 | 300 ± 50 | 120 ± 10 | 37 ± 11 | 3000 ± 1100 | 12 ± 1  |
| n-Val-pNA       | 38 ± 2 | 600 ± 50 | 63 ± 6   | 7 ± 1  | 1100 ± 2000 | 7 ± 1  |
| n-Val-pNA       | 17.4 ± 0.5 | 56 ± 5 | 310 ± 29  | 28 ± 2   | 420 ± 60 | 68 ± 1  |
| Pyro-EFK-pNA    | 36.8 ± 0.7 | 154 ± 10 | 240 ± 20  | 48 ± 4   | 2200 ± 200 | 22 ± 2  |
| Tosyl-GPK-pNA   | 26 ± 2 | 482 ± 50 | 50 ± 10   | 23 ± 7   | 8000 ± 3000 | 2.9 ± 0.9 |
| Tosyl-GPR-pNA   | 44 ± 3 | 1000 ± 100 | 44 ± 6 | 5 ± 2   | 2000 ± 1000 | 3 ± 1   |
| Pyro-EFR-pNA    | 87 ± 4 | 390 ± 40 | 220 ± 30  | 50 ± 20  | 5000 ± 2000 | 12 ± 4  |
| n-IPR-pNA       | 31 ± 2 | 3500 ± 400 | 8.7 ± 0.8 | 4.7 ± 0.7 | 2900 ± 700 | 1.6 ± 0.3 |
| PGR-pNA         | 1.2 ± 0.3 | 4100 ± 1400 | 0.29 ± 0.10 | 0.14 ± 0.06 | 3300 ± 1700 | 0.04 ± 0.02 |
| CBO-RGR-pNA     | 8.4 ± 0.2 | 120 ± 10 | 70 ± 6   | 2.3 ± 0.6 | 600 ± 300 | 4 ± 2   |
| EGR-pNA         | 18 ± 2 | 9000 ± 2000 | 1.9 ± 0.4 | 0.6 ± 0.1 | 4000 ± 1000 | 0.18 ± 0.05 |
| GR-pNA          | 0.11 ± 0.03 | 7000 ± 3000 | 0.016 ± 0.008 | NA  | NA  | NA  |

Fig. 7. Comparison of kinetic parameters for hydrolysis of tripeptide-pNA substrates by SKPG* and SKPm. A, the fractional difference in $k_i$ between SK[Lys]PG* and SKPm* ($k_i$SK[Lys]PG*/$k_i$SKPm*). B, the fractional difference in $K_m$ between SK[Lys]PG* and SKPm* ($K_m$SK[Pm]* − $K_m$SK[Pm])/$K_m$SK[Pm]*) is shown for hydrolysis of the indicated 11 tripeptide-pNA substrates. The solid bars represent substrates containing Lys in the P1 position, and the open bars refer to those containing Arg at P1.

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the catalytic site, which is in a deep cavity surrounded by SK in contribution substantially to the loss of specificity with SK binding. The affinities of SK binding and conformational activation of native [Lys]Pg were both decreased in [Glu]Pg binding. The affinities of SK binding and conformational activation of native [Lys]Pg were both ~12-fold higher than [Glu]Pg, and this difference was lost when lysine-binding sites were blocked with 6-AHA. The kinetically measured SK affinities for [Glu]Pg and [Lys]Pg activation in the presence of 6-AHA were equivalent and in good agreement with the affinities determined by competitive binding. The source of the differential contribution of lysine-binding sites to conformational activation of [Glu]Pg and [Lys]Pg is thought to be the different conformations of these Pg forms and the associated differences in accessibility of lysine-binding sites. At physiological chloride ion concentration, [Glu]Pg is in a compact conformation, maintained by interactions between the N-terminal 77-residue peptide and lysine-binding sites in kringle 4 and 5 (4–6, 54). Cleavage of the N-terminal peptide by Pm to form [Lys]Pg is accompanied by a shift to the extended conformation and exposure of unoccupied lysine-binding sites (5, 7, 13). In the interaction of SK with [Glu]Pg, 6-AHA has no significant effect because lysine-binding sites are inaccessible or blocked in both the compact conformation and the extended conformation produced by 6-AHA binding, respectively. The increased affinity of SK for [Lys]Pg and the accompanying increased affinity of conformational activation was reduced to that of [Glu]Pg in the presence of 6-AHA. Thus, the compact-extended conformational change in [Glu]Pg by itself has apparently little effect on SK binding or conformational activation but indirectly enhances affinity and activation through increased lysine-binding site interactions. This was substantiated by the observation here that SK bound and conformationally activated [Glu]Pg in the extended conformation formed at low chloride concentrations with high affinity, which is due to lysine-binding site interactions with the extended form (33). On the basis of the large differences in affinity between the compact and extended forms of [Glu]Pg, previous studies (33) concluded that SK binding is accompanied by conversion of [Glu]Pg to the extended form in the SK-[Glu]Pg$^*$ complex. The partial reduction in the affinity of SK for activating [Lys]Pg by 6AHA, the lack of an effect for [Glu]Pg, and the similar chromogenic substrate activities of the SK-Pg$^*$ complexes in the absence and presence of 6-AHA demonstrate that lysine-binding site interactions linked to the compact-extended equilibrium enhance but are not required for Pg binding and conformational activation. The specificity of conformationally activated Pg in the transiently formed SK-Pg$^*$ complex for tripeptide-pNA substrates was distinctly different from the specificity of free Pm and SK-Pm characterized previously for the same panel of substrates (21). In terms of specificity constants, Pm was the most specific; the specificity of SK-Pm was unaffected or reduced 2.6–10-fold for the majority of substrates (21), and SK-Pg$^*$ was the least specific, with reductions of 18–130-fold compared with Pm. As shown previously for SK-Pm, increases in $K_m$ contribute substantially to the loss of specificity with SK binding. This may be the result of reduced access of substrates to the catalytic site, which is in a deep cavity surrounded by SK in the SK-micro-Pm structure (20, 55). However, binding of SK to Pm also results in a uniquely different specificity for substrate residues in the P2 position, whereas the marginally higher specificity of Pm for substrates with Lys compared with Arg at P1 is unaffected (21). By contrast, comparison of SK-Pg$^*$ and SK-Pm demonstrated differences in specificity dependent on P1. Substrates with Lys at P1 were primarily different in SK-Pg$^*$ because of increases in $K_m$, with modest changes in $k_{cat}$. $K_m$ for substrates with Arg at P1, with one exception, was not greatly affected, whereas $k_{cat}$ was consistently decreased. These results indicate that the S1 specificity subsite in SK-Pg$^*$ and SK-Pm is distinctly different, characterized by a loss of apparent substrate affinity for P1 Lys substrates and decreased rates of catalytic turnover of P1 Arg substrates. The uniquely different specificities of SK-Pm and the conformationally activated catalytic site in SK-Pg$^*$ were not correlated with a preference of either complex for cleavage of the Pro-Gly-Arg activation sequence in Pg, consistent with exosite-mediated Pg substrate binding dominating Pg substrate recognition by both complexes (21). The difference in substrate specificity of SK-Pg$^*$ and SK-Pm suggests the possibility that the catalytic site is incompletely formed in the conformationally activatedzymogen. However, it is also possible that this difference is due to differences between the enzyme and zymogen in their interactions with SK domains that affect the catalytic site. Three components have been postulated to contribute to the mechanism of conformational Pg activation. Studies demonstrating a critical role of the N terminus of SK in conformational activation support the “molecular sexuality” mechanism (34, 56, 57). In this mechanism, insertion of the SK N terminus into the N-terminal binding cleft in the Pg catalytic domain and salt bridge formation with Asp$^{194}$ (chymotrypsin numbering) triggers the activating conformational change. Other studies support a role for interactions of the SK $\gamma$-domain with Pg resulting in reorientation of Lys$^{556}$ to form the critical salt bridge (20, 55, 58). The contribution of these two mechanisms to conformational activation has not been resolved. In either case, the ~800-fold higher affinity of SK for the activated catalytic domain of Pm compared with [Lys]Pg is also thought to contribute to nonproteolytic activation by stabilization of the activated conformation (21, 34). The present studies demonstrate that the molecular events in conformational activation are initiated by rapid and reversible SK binding. Rapid-reaction kinetics of SK binding to fluorescein-labeled [Glu]Pg and [Lys]Pg demonstrated biphasic reactions, indicating that at least two binding or conformational change reactions occur and can be resolved on the conformational activation pathway. The effect of 6-AHA on the kinetics of SK binding to [Glu]Pg and [Lys]Pg showed that the kinetics for [Glu]Pg were unaffected, whereas the rate of the fast phase was decreased for [Lys]Pg to that observed for [Glu]Pg. These results parallel the effects of 6-AHA on SK binding and conformational activation and indicate that the fast phase of SK binding to [Lys]Pg is linked to interactions with lysine-binding sites on Pg. One of these fast reactions may correspond to the isomerization of the SK-Pg$^*$ complex at low chloride concentrations described previously (22). The results indicate that further rapid-reaction kinetic studies should enable resolution of the individual molecular events on the pathway of conformational Pg activation by SK. The mechanism of coupling of the conformational activation process characterized here to proteolytic Pg activation is addressed in the companion paper (37).

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Resolution of Conformational Activation in the Kinetic Mechanism of Plasminogen Activation by Streptokinase
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