Streptokinase Binds Preferentially to the Extended Conformation of Plasminogen through Lysine Binding Site and Catalytic Domain Interactions†

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ABSTRACT: Binding of streptokinase (SK) to plasminogen (Pg) activates the zymogen conformationally and initiates its conversion into the fibrinolytic proteinase, plasmin (Pm). Equilibrium binding studies of SK interactions with a homologous series of catalytic site-labeled fluorescent Pg and Pm analogues were performed to resolve the contributions of lysine binding site interactions, associated changes between extended and compact conformations of Pg, and activation of the proteinase domain to the affinity for SK. SK bound to fluorescein-labeled [Glu]Pg_1 and [Lys]Pg_1 with dissociation constants of 624 ± 112 and 38 ± 5 nM, respectively, whereas labeled [Lys]Pm_1 bound with a 57000-fold tighter dissociation constant of 11 ± 2 pM. Saturation of lysine binding sites with 6-aminohexanoic acid had no effect on SK binding to labeled [Glu]Pg_1, but weakened binding to labeled [Lys]Pg_1 and [Lys]Pm_1 31- and 20-fold, respectively. At low Cl^- concentrations, where [Glu]Pg assumes the extended conformation without occupation of lysine binding sites, a 23-fold increase in the affinity of SK for labeled [Glu]Pg_1 was observed, which was quantitatively accounted for by expression of new lysine binding site interactions. The results support the conclusion that the SK affinity for the fluorescent Pg and Pm analogues is enhanced 13−16-fold by conversion of labeled [Glu]Pg to the extended conformation of the [Lys]Pg derivative as a result of lysine binding site interactions, and is enhanced 3100−3500-fold further by the increased affinity of SK for the activated proteinase domain. The results imply that binding of SK to [Glu]Pg results in transition of [Glu]Pg to an extended conformation in an early event in the SK activation mechanism.

Activation of human plasminogen (Pg) by streptokinase (SK)[1] generates the fibrinolytic proteinase, plasmin (Pm), through a unique mechanism that is the basis for the clinical use of SK as a thrombolytic drug (*1*, *2*). SK activates Pg by inducing catalytic activity in the Pg zymogen through a conformational change and, subsequently, by proteolytic activation of free Pg to Pm catalyzed by the active SK−Pg complex (*3*−*7*). SK also binds to Pm and converts the substrate specificity of the proteinase into that of a specific Pg activator (*7*−*9*). Native [Glu]Pg consists of an amino-terminal 77-residue peptide, followed by five homologous kringle domains and a serine proteinase catalytic domain containing the single Arg^{561}−Val^{562} activation cleavage site (*2*, *10*, *11*). Pg circulates in blood as a mixture of two natural carbohydrate variants, designated forms 1 and 2, which have been reported to differ in their properties as substrates of plasminogen activators (*12*−*15*). Kringle domains 1, 4, and 5 of Pg contain binding sites for carboxy-terminal lysine residues, which mediate binding of Pg and Pm to lysine residues of fibrin, and other physiological protein interactions (*16*−*21*). The X-ray crystal structure of SK bound to the isolated catalytic domain of Pm shows that SK consists of three "β-grasp-like" domains which form a "three-sided crater" around the Pm active site (*22*). This interaction of SK with full-length Pm results in expression of an exosite that mediates specific recognition of Pg as a substrate for proteolytic activation by the SK−Pm complex (*23*).

The mechanism of fibrinolysis is regulated by conformational changes between extended and compact conformations of Pg that are linked to lysine binding site interactions (*1*, *2*, *16*, *24*, *25*). Binding of the amino-terminal peptide of [Glu]-Pg to lysine binding sites on kringles 4 and 5 within the same molecule maintains [Glu]Pg in a compact spiral conformation in which the lysine binding sites are occupied and Pg activation is inhibited (*26*, *27*). As a result of Pm formation during Pg activation, the amino-terminal 77-residue peptide of [Glu]Pg is cleaved by Pm to form [Lys]-Pg, which adopts an extended conformation due to the loss of lysine binding site interactions with the peptide, and is activated at an accelerated rate (*28*−*30*). Disruption of the internal lysine binding site interactions in [Glu]Pg with lysine analogues such as 6-aminohexanoic acid (6-AHA) also shifts

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[1] Abbreviations: [Glu]Pg_1 and [Glu]Pg_2, carbohydrate forms 1 and 2 of native plasminogen, respectively; [Lys]Pg_1 and [Lys]Pg_2, forms 1 and 2 of Pg lacking the 77 amino-terminal residues, respectively; [Lys]-Pm_1 and [Lys]Pm_2, plasmin forms 1 and 2, respectively; SK, streptokinase; 6-AHA, 6-aminohexanoic acid; FFR-CH_2Cl, (D-Phe)-Phe-Arg-CH_2Cl; ATA-FFR-CH_2Cl, N^{α}-[(acetylthio)acetyl]-(D-Phe)-Phe-Arg-CH_2Cl; [5-F]FFR-[Glu]Pg, [5-F]FFR-[Lys]Pg, and [5-F]FFR-[Lys]Pm, Pg and Pm species labeled with ATA-FFR-CH_2Cl and 5-(iodoacetamido)fluorescein; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PEG, polyethylene glycol 8000; SDS, sodium dodecyl sulfate.

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the Pg equilibrium to an extended conformation (*24*, *25*, *31*, *32*), which is similar but not identical to the extended form of [Lys]Pg (*32*). In an opposing regulatory process, chloride ions bind specifically to [Glu]Pg, shifting the conformational equilibrium to the compact conformation at physiological levels (*33*−*36*). Kinetic studies of the effects of 6-AHA and Cl^- on Pg activation by urokinase and tissue-type plasminogen activator have shown that the rates are accelerated by 6-AHA and inhibited by Cl^-, demonstrating that the extended conformation is the favored substrate of these activators (*33*, *35*). In contrast, activation of [Glu]Pg by SK is inhibited by both 6-AHA (*37*) and Cl^- (*34*), indicating a different and poorly understood mechanism of involvement of lysine binding sites, in which the conformation of Pg affects the formation of the SK−Pg complex and/or its activity in Pg activation.

To delineate the pathway of Pg activation by SK, the roles of Pg conformational changes and lysine binding sites in modulating the affinities of interactions of SK with Pg and Pm species must be quantitatively characterized in equilibrium binding studies. Results of such previous studies, however, vary considerably, with reported estimates of the affinity of SK for various forms of Pg and Pm that range over 3−4 orders of magnitude (*38*−*44*). Binding studies employing Pg and Pm derivatives that were labeled at the active site with the fluorescence probe, 2-anilinonaphthalene-6-sulfonic acid, showed that SK interacts with labeled [Lys]-Pg with an affinity that is higher than that of the [Glu]Pg homologue, and the affinity for labeled [Lys]Pg, but not the affinity for labeled [Glu]Pg, was dependent on lysine binding sites (*40*). This finding was not supported, however, by results of other studies using surface plasmon resonance, which reported a dependence of SK affinity for [Glu]Pg on lysine binding sites (*39*). Similar disagreement exists for plasmin, where equivalent affinities of Pg and Pm for SK and a vastly increased affinity for Pm have both been reported (*23*, *38*, *41*−*43*). The sources of these discrepancies are not entirely understood, but may be related to the use of surface-adsorbed proteins in some previous binding studies, coupled with the possible effects of proteolytic formation of [Lys]Pg and [Lys]Pm on SK binding, processes which can be eliminated in the fluorescence probe approach.

In the fluorescence studies presented here, the interactions of SK with a homologous series of active site-labeled fluorescein derivatives of both carbohydrate variants of [Glu]-Pg, [Lys]Pg, and [Lys]Pm were compared comprehensively, to gain further insight into the role of changes in SK binding affinity in the mechanism of activation of Pg. SK bound labeled [Lys]Pm with extremely high affinity, and the affinities of SK for labeled [Lys]Pg and [Glu]Pg were 3100−3500-fold and 41000−57000-fold lower, respectively. No significant differences were observed in SK binding to the fluorescent derivatives of the two carbohydrate variants of Pg and Pm. Characterization of the effects of 6-AHA and chloride ion on SK affinity for the labeled Pg and Pm species showed that SK binds with a 23-fold higher affinity to the extended conformation of labeled [Glu]Pg, and that the enhanced affinity is due entirely to the increased availability of lysine binding sites in the extended conformation. Proteolytic activation of the catalytic domain of the Pg analogue increased the SK affinity 3100−3500-fold further, independent of lysine binding site interactions. Preferential binding of SK to the extended conformation of labeled Pg implies that transition of [Glu]Pg from the compact to an extended conformation through lysine binding site interactions accompanies formation of the SK−Pg complex as an early event in the activation mechanism.

EXPERIMENTAL PROCEDURES

Protein Purification and Characterization. [Glu]Pg carbohydrate forms 1 and 2 were purified from human plasma (*12*). [Lys]Pm was prepared by activation of 10 μM [Glu]-Pg with 90 units/mL urokinase (Calbiochem) in 10 mM MES, 10 mM HEPES, 0.15 M NaCl, 20 mM 6-AHA, and 1 mg/mL PEG at pH 7.4 and 25 °C and isolated by affinity chromatography on soybean trypsin inhibitor agarose by the published method (*40*, *45*). Purified [Lys]Pm was dialyzed against 5 mM HEPES, 0.3 M NaCl, 10 mM 6-AHA, and 1 mg/mL PEG at pH 7.0 and 4 °C. Native SK was obtained from Diapharma and purified by affinity chromatography on a column (1.5 cm × 9 cm) of Pm immobilized on iodoacetyl-(3,3′-iminobispropylamine)agarose (Pierce Sulfolink) through its active site with ATA-FFR-CH_2Cl (5.5 mg of Pm coupled/mL of gel) (*40*, *45*).

Protein concentrations were determined by absorbance at 280 nm using the following absorption coefficients (M^{-1} cm^{-1}) and molecular weights: SK, 0.95 and 47 000 (*46*, *47*); [Glu]Pg, 1.69 and 92 000; [Lys]Pg, 1.69 and 84 000; and Pm, 1.9 and 84 000 (*12*, *23*). Pm preparations were 71−84% active as determined by titration with *p*-nitrophenyl *p*-guanidinobenzoate or fluorescein mono-*p*-guanidinobenzoate (*23*). Chromogenic substrate activity was measured by the initial rates of hydrolysis of 200 μM H-D-Val-Leu-Lys-*p*-nitroanilide at 405 nm in polyethylene glycol 20000-coated cuvettes.

Gel Electrophoresis. SDS 4−15% polyacrylamide gradient gels (Bio-Rad) were stained with GELCODE Blue (Pierce), or fluorescence was visualized with a 300 nm transilluminator.

Preparation of Active Site-Labeled [Lys]Pm. ATA-FFR-CH_2Cl was prepared as described previously (*48*, *49*). ATA-FFR-[Lys]Pm was prepared by incubation of a 5-fold excess of inhibitor with 10−15 μM Pm in 0.1 M HEPES, 0.3 M NaCl, 1 mM EDTA, 10 mM 6-AHA, and 1 mg/mL PEG at pH 7.0 and 25 °C for 30−60 min, until inactivation was >99.9% complete. Excess inhibitor was removed by dialysis against >400 volumes of 50 mM HEPES, 0.3 M NaCl, and 1 mM EDTA at pH 7.0 and 4 °C. Quantitation of the incorporation of ATA-FFR-CH_2Cl from the NH_2OH-initiated burst of thiol measured with 5,5′-dithiobis(2-nitrobenzoic acid) (*49*) gave stoichiometries of 1.0−1.1 mol of thioester/mol of Pm active sites, with no significant free thiol (<1%) detected. For labeling with fluorescein, 1−8 mL reactions including 4−8 μM ATA-FFR-Pm and a 5−10-fold excess of 5-(iodoacetamido)fluorescein (Molecular Probes) were initiated by addition of 0.1 M NH_2OH and the mixtures incubated for 1 h at 25 °C in the dark. Excess dye was removed by chromatography on a 9 or 25 mL column of Sephadex G-25 (superfine), followed by dialysis in the dark against >400 volumes of the buffer used in the experiments. Quantitation of fluorescein incorporation as described previously (*48*) gave stoichiometries of 0.9−1.1 mol of probe/mol of Pm active sites.

Preparation of Catalytic Site-Labeled Pg. [Glu]Pg and [Lys]Pg were labeled at the catalytic site by a modification of the method described previously (*40*). The SK−Pg complex was inactivated by incubation of 25 μM [Glu]Pg with 50 μM SK in 1 M HEPES, 0.3 M NaCl, and 1 mM EDTA at pH 7.0 and 25 °C for 3 h in the presence of 400 μM ATA-FFR-CH_2Cl. Reaction progress was monitored by loss of chromogenic substrate activity, and the reaction mixture was chromatographed on Sephadex G-25 (superfine) (1.5 cm × 26 cm) to remove the excess inhibitor. Labeling of the ATA-FFR-(SK−Pg) complex was accomplished by addition of 0.1 M NH_2OH to a mixture of 8−15 μM complex and a 10−14-fold excess of 5-(iodoacetamido)fluorescein in the above 1 M HEPES buffer at 25 °C. After incubation for 1 h in the dark, the reaction was stopped with 50 μM FFR-CH_2Cl, and excess label was removed by chromatography on Sephadex G-25 (superfine) (1.5 cm × 26 cm). To separate labeled Pg from SK, the labeled complex was chromatographed twice on a column of active site-immobilized Pm at a flow rate of 2 mL/h in 0.1 M HEPES, 0.1 M NaCl, 10 mM 6-AHA, 1 mM EDTA, and 0.02% NaN_3 at pH 7.4 and room temperature. Labeled Pg was purified further by chromatography on an SK-Affigel column (1.5 cm × 12.5 cm) prepared as recommended by the manufacturer (Bio-Rad; 4−5 mg coupled/mL of gel). In the above HEPES buffer containing 20 mM 6-AHA at 25 °C, residual labeled SK−Pg and SK−Pm complexes did not bind to the column. Subsequent elution with a 5−10 column volume gradient of NaSCN up to 3 M in the same buffer yielded labeled Pg in the first peak, separated from later-eluting, labeled [Lys]Pm. Fractions containing the labeled Pg were pooled, concentrated with a YM-30 ultrafiltration membrane, and dialyzed against >3500 volumes of 0.1 M HEPES, 0.1 M NaCl, 1 mM EDTA, and 1 mg/mL PEG at pH 7.4 and 4 °C in the dark. Labeled [Lys]Pg was prepared as described for [Glu]Pg, except that initially 40 μM [Glu]Pg was converted to [Lys]-Pg by incubation with 2−5 μM Pm in 50 mM tris-(hydroxymethyl)aminomethane hydrochloride, 20 mM L-lysine, and 0.1 M NaCl at pH 9.0 and 25 °C for 30 min. [Lys]Pg was incubated with 10 μM FFR-CH_2Cl at 25 °C, and dialyzed into the 1 M HEPES reaction buffer prior to labeling and purification as described above.

Fluorescence Studies. Fluorescence measurements were taken with an SLM 8100 spectrofluorometer in the ratio mode, using acrylic cuvettes coated with polyethylene glycol 20000. Fluorescence titrations were performed with excitation and emission maximum wavelengths of 500 and 516 nm, respectively (8 or 16 nm band-pass), which were determined from spectra (4 nm band-pass) of labeled Pg and Pm in the absence and presence of near-saturating (5 μM) SK. Fluorescence titrations were performed by sequential addition of small volumes of SK to a cuvette containing labeled Pg or Pm in 50 mM HEPES, 0.125 M NaCl or 1 mM NaCl with 124 mM sodium acetate, 1 mM EDTA, 1 mg/mL PEG, 1 mg/mL bovine serum albumin, and 1 μM FFR-CH_2Cl with or without 100 mM 6-AHA (Fluka) at pH 7.4 and 25 °C, as indicated. Fluorescence changes were measured after equilibration for 5−10 min, and expressed as the fractional change in the initial fluorescence [$(F_{obs} - F_o)/F_o = \Delta F/F_o$]. Blank cuvettes lacking labeled protein were titrated in parallel, and the background was subtracted. Fluorescence changes were confirmed in control experiments

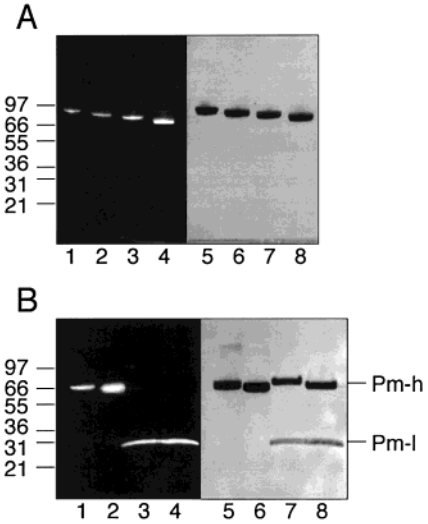

FIGURE 1: Characterization of [5-F]FFR-labeled Pg and Pm. (A) SDS gels of [5-F]FFR-labeled Pg visualized by fluorescence (lanes 1−4) and Coomassie blue staining (lanes 5−8). Results are shown for nonreduced, 5 μg samples of [5-F]FFR-labeled $[Glu]Pg_1$ (lanes 1 and 5), $[Glu]Pg_2$ (lanes 2 and 6), $[Lys]Pg_1$ (lanes 3 and 7), and $[Lys]Pg_2$ (lanes 4 and 8). (B) Fluorescence (lanes 1−4) and protein-stained (lanes 5−8) SDS gel of [5-F]FFR-$[Lys]Pm_1$ (lanes 1, 3, 5, and 7) and [5-F]FFR-$[Lys]Pm_2$ (lanes 2, 4, 6, and 8) under nonreducing (lanes 1, 2, 5, and 6) and reducing conditions (lanes 3, 4, 7, and 8).

to be rapidly achieved, reproducible, and stable with time. Titrations were analyzed by nonlinear least-squares fitting of the quadratic binding equation, with the maximum fluorescence change ($\Delta F_{max}/F_o$) and dissociation constant (K_D) as the fitted parameters. The stoichiometric factor (n) was fixed at 1 or at the value previously determined for the interaction. Least-squares fitting was performed with Scientist software (MicroMath). Uncertainties in reported parameters are ±2 standard deviations.

RESULTS

Characterization of [5-F]FFR-Labeled Pg and Pm. A homologous series of catalytic site-labeled fluorescent Pg and Pm analogues was prepared to investigate the effects of Pg and Pm conformation on SK binding. Both carbohydrate variants of [Lys]Pm were labeled specifically by active site-directed alkylation of the catalytic site histidine residue with ATA-FFR-CH_2Cl and subsequent modification of the thiol group generated on the inhibitor with 5-(iodoacetamido)-fluorescein (*48*, *49*). By taking advantage of the reversible conformational activation of Pg induced by SK, we similarly labeled and purified both carbohydrate variants of [Glu]Pg and [Lys]Pg (*40*). All fluorescent protein preparations were homogeneous as determined by SDS gel electrophoresis and were specifically labeled in the catalytic domain with the fluorophore (Figure 1). Stoichiometries of probe incorporation were 0.9−1.1 mol of probe/Pm active sites and 0.8−1.0 mol of probe/mol of Pg.

Comparison of fluorescence emission spectra of the fluorescein-labeled proteins in the absence and presence of saturating (5 μM) SK showed no significant spectral shifts (≤2 nm) associated with SK binding to labeled [Glu]Pg, [Lys]Pg, or [Lys]Pm (Figure 2). When compared at the same fluorescein concentration, the fluorescence intensities of labeled [Glu]Pg and [Lys]Pg were indistinguishable, and that

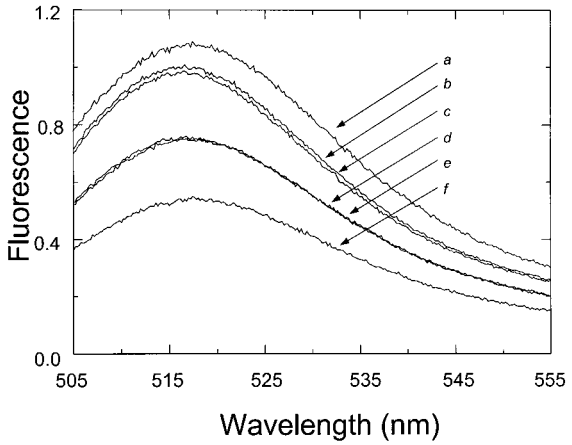

FIGURE 2: Effect of SK on the fluorescence emission spectra of [5-F]FFR-[Glu]Pg, [5-F]FFR-[Lys]Pg, and [5-F]FFR-[Lys]Pm. Fluorescence emission spectra with excitation at 500 nm of 50 nM [5-F]FFR-[Lys]Pm_2 (a), [5-F]FFR-[Glu]Pg_2 (b), and [5-F]FFR-[Lys]-Pg_2 (c) are shown. Spectra in the presence of 5 μM SK were of [5-F]FFR-[Glu]Pg_2 (d), [5-F]FFR-[Lys]Pg_2 (e), and [5-F]FFR-[Lys]-Pm_2 (f). Spectra were collected in $I = 0.15$ M, pH 7.4 buffer containing 125 mM Cl^- as described in Experimental Procedures and normalized to the same fluorescein concentration for comparison.

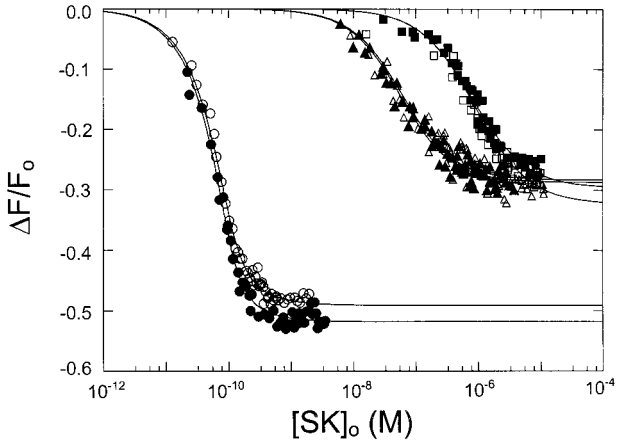

FIGURE 3: SK binding to [5-F]FFR-[Glu]Pg, [5-F]FFR-[Lys]Pg, and [5-F]FFR-[Lys]Pm. The fractional changes in fluorescence ($\Delta F/F_o$) are plotted as a function of total concentration of SK ($[SK]_o$) for titrations of carbohydrate forms 1 (●, ▲, and ■) and 2 (○, △, and □) of 30 nM [5-F]FFR-[Glu]Pg (■ and □), 15 nM [5-F]FFR-[Lys]Pg (▲ and △), and 75 pM [5-F]FFR-[Lys]Pm (● and ○) in buffer containing 125 mM Cl^-. Solid lines represent least-squares fits of the quadratic binding equation to the data with the parameters listed in Table 1 and the text. Fluorescence titrations were performed and analyzed as described in Experimental Procedures.

of labeled [Lys]Pm was only ~8% higher. Binding of SK, however, produced indistinguishable decreases in fluorescence intensity of 28−32% for [5-F]FFR-[Glu]Pg_2 and [5-F]-FFR-[Lys]Pg_2, and the dissimilar value of 49−52% for [5-F]FFR-[Lys]Pm_2. These results indicated that the fluorescein probe was relatively insensitive to activation of the labeled Pg catalytic site upon Pm formation, but reported significant differences between other perturbations of the catalytic site of the Pg and Pm analogues that were induced specifically by SK binding.

Binding of SK to [5-F]FFR-[Glu]Pg, [5-F]FFR-[Lys]Pg, and [5-F]FFR-[Lys]Pm. Analysis of titrations with SK of both carbohydrate forms of [5-F]FFR-[Glu]Pg, [5-F]FFR-[Lys]Pg, and [5-F]FFR-[Lys]Pm in $I = 0.15$ M, pH 7.4 buffer containing 125 mM NaCl demonstrated vastly different affinities for SK, and insignificant differences between the two carbohydrate variants of these species (Figure 3). SK bound to carbohydrate variants 1 and 2 of [5-F]FFR-[Lys]-Pm with dissociation constants of 11 ± 2 and 14 ± 2 pM and maximum fluorescence changes of −52 ± 1 and −49 ± 1%, respectively. Strikingly, [5-F]FFR-[Lys]Pg forms 1 and 2 bound SK with 3100−3500-fold lower affinity than labeled [Lys]Pm, yielding dissociation constants of 38 ± 5 and 44 ± 9 nM and smaller fluorescence changes of −29 ± 1 and −28 ± 1%, respectively. SK binding to [5-F]FFR-[Glu]Pg was the lowest-affinity interaction, with a 13−16-fold lower affinity than labeled [Lys]Pg. Indistinguishable dissociation constants of 624 ± 112 and 578 ± 81 nM and maximum fluorescence changes of −30 ± 1 and −32 ± 1% were obtained for carbohydrate forms 1 and 2 of fluorescein-labeled [Glu]Pg, respectively. Overall, the affinity of SK for [5-F]FFR-[Lys]Pm was enhanced 41000−57000-fold compared to that for [5-F]FFR-[Glu]Pg (Table 1). These results demonstrated a large increase in the affinity for SK accompanying conversion of labeled [Glu]Pg to [Lys]Pg, and an even larger enhancement due to cleavage and activation of the proteinase domain.

Involvement of Lysine Binding Sites in the Interactions of SK with [5-F]FFR-[Glu]Pg, [5-F]FFR-[Lys]Pg, and [5-F]-FFR-[Lys]Pm. To resolve the contributions of lysine binding sites to the observed differences in affinity of SK for the Pg and Pm analogues, the effect of a saturating concentration of 6-AHA (100 mM) on binding of SK was characterized, initially in buffer containing 125 mM Cl^-. The expected effects of 6-AHA binding on the conformations of [5-F]FFR-labeled [Glu]Pg, [Lys]Pg, and [Lys]Pm are summarized schematically in Figure 4 (*24*−*27*, *31*, *32*). The affinities of SK for [5-F]FFR-[Lys]Pg_1 and [5-F]FFR-[Lys]Pm_1 were reduced similarly by saturating 6-AHA, 31- and 20-fold, respectively, whereas the affinity of SK for [5-F]FFR-[Glu]-Pg_1 was unaffected (Figure 5 and Table 1). This differential effect of 6-AHA normalized the difference in affinity between labeled [Glu]Pg and [Lys]Pg, indicating that lysine binding site interactions accounted for this difference (Table 1). The fluorescence intensity changes for SK binding were minimally (≤9%) affected by the presence of 6-AHA, showing no major effects of the compact to extended Pg conformational change accompanying saturation of lysine binding sites on the environment of the active site-bound probe. Titrations of carbohydrate forms 1 and 2 of labeled Pg and Pm with SK gave similar results, with subtle differences (≤4%) in the amplitudes of the fluorescence changes (Table 1). The results demonstrated that lysine binding site interactions facilitated SK binding to [5-F]FFR-[Lys]Pg and [5-F]FFR-[Lys]Pm under conditions where they were in the extended conformation and the lysine binding sites were initially unoccupied. The contrasting absence of an effect of 6-AHA on [5-F]FFR-[Glu]Pg was consistent with the inaccessibility of lysine binding sites on kringles 4 and 5 in the compact conformation (*26*, *27*) and their inaccessibility in the extended conformation produced by saturation of lysine binding sites with 6-AHA (*24*, *25*, *31*, *32*) (Figure 4).

Role of Cl^--Dependent Conformational Equilibria in SK Binding to [5-F]FFR-Labeled Pg and Pm. The effect on SK

Table 1: Parameters for Streptokinase Binding to Fluorescein-Labeled Plasmin and Plasminogen Analogues[a]

	K_D (nM)				$\Delta F_{max}/F_o$ (%)			
	no 6-AHA		100 mM 6-AHA		no 6-AHA		100 mM 6-AHA	
protein	1 mM Cl^-	125 mM Cl^-	1 mM Cl^-	125 mM Cl^-	1 mM Cl^-	125 mM Cl^-	1 mM Cl^-	125 mM Cl^-
[5-F]FFR-[Lys]Pm_1	0.019 ± 0.004	0.011 ± 0.002	0.21 ± 0.03	0.22 ± 0.03	-54 ± 1	-52 ± 1	-58 ± 2	-48 ± 1
[5-F]FFR-[Lys]Pm_2	0.013 ± 0.003	0.014 ± 0.002	0.14 ± 0.02	0.21 ± 0.02	-48 ± 1	-49 ± 1	-54 ± 2	-53 ± 1
[5-F]FFR-[Lys]Pg_1	10 ± 2	38 ± 5	281 ± 33	1174 ± 164	-29 ± 1	-29 ± 1	-28 ± 1	-25 ± 1
[5-F]FFR-[Lys]Pg_2	8 ± 2	44 ± 9	186 ± 36	557 ± 89	-25 ± 1	-28 ± 1	-25 ± 1	-23 ± 1
[5-F]FFR-[Glu]Pg_1	11 ± 4	624 ± 112	252 ± 29	578 ± 85	-29 ± 1	-30 ± 1	-29 ± 1	-28 ± 1
[5-F]FFR-[Glu]Pg_2	13 ± 4	578 ± 81	285 ± 31	606 ± 72	-26 ± 1	-32 ± 1	-24 ± 1	-23 ± 1

[a] Binding parameters were determined by analysis of titrations of the fractional change in fluorescence vs total streptokinase concentration as described in Experimental Procedures to obtain the dissociation constants (K_D) and maximum fluorescence changes ($\Delta F_{max}/F_o$) listed here. The buffers were 50 mM HEPES, 125 mM NaCl, 1 mM EDTA, and 1 mg/mL PEG (pH 7.4) or 50 mM HEPES, 1 mM NaCl, 124 mM sodium acetate, 1 mM EDTA, and 1 mg/mL PEG (pH 7.4) with 100 mM 6-AHA, as indicated.

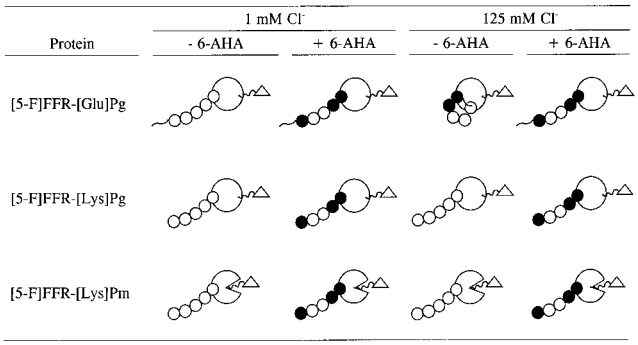

FIGURE 4: Summary of effects of 6-AHA and Cl^- on conformations of [5-F]FFR-[Glu]Pg, [5-F]FFR-[Lys]Pg, and [5-F]FFR-[Lys]Pm. Labeled [Glu]Pg is represented as an amino-terminal peptide (squiggle), followed by five kringles (small circles), and the catalytic domain (large circle) containing the covalently linked fluorescence probe (triangle). Black circles represent kringles with occupied lysine binding sites. At 125 mM Cl^-, [Glu]Pg adopts a compact conformation in which the amino-terminal peptide interacts with kringles 4 and 5. Disruption of this interaction by low Cl^- concentrations, competition for lysine binding sites by 6-AHA, or proteolytic cleavage of the amino-terminal peptide results in extended conformations.

binding of the conformational change of Pg from the compact to extended form induced by lowering the Cl^- concentration (see Figure 4) was assessed by comparison of SK binding to [5-F]FFR-labeled Pg and Pm in 1 mM Cl^- (Figure 6) with the above results in 125 mM Cl^-. In experiments at 1 mM Cl^-, a constant ionic strength ($I = 0.15$ M) was maintained by the presence of sodium acetate, an anion which does not similarly affect the Pg conformation (*34*). The low chloride concentration represented ~10% of the dissociation constant (*34*). The near absence of chloride did not significantly affect the binding of SK to [5-F]FFR-[Lys]Pm_1 and modestly increased the binding affinity of [5-F]FFR-[Lys]Pg_1 for SK to 10 ± 2 nM, a 3.8-fold increase in affinity over that in 125 mM Cl^- (Figure 6 and Table 1). In contrast to these small effects, [5-F]FFR-[Glu]Pg exhibited a marked dependence on Cl^-, with SK binding 44−57-fold tighter in 1 mM Cl^- (Table 1). The maximum changes in fluorescence accompanying SK binding were slightly reduced (≤6%) in 1 mM Cl^-. No significant differences in the affinities of SK for carbohydrate variants 1 and 2 were observed (Table 1). These results showed that the affinity for SK was increased by shifting the conformation of [5-F]FFR-[Glu]Pg toward the extended form.

Role of Lysine Binding Site Interactions in Cl^--Dependent Binding of SK to [5-F]FFR-Labeled Pg and Pm. To

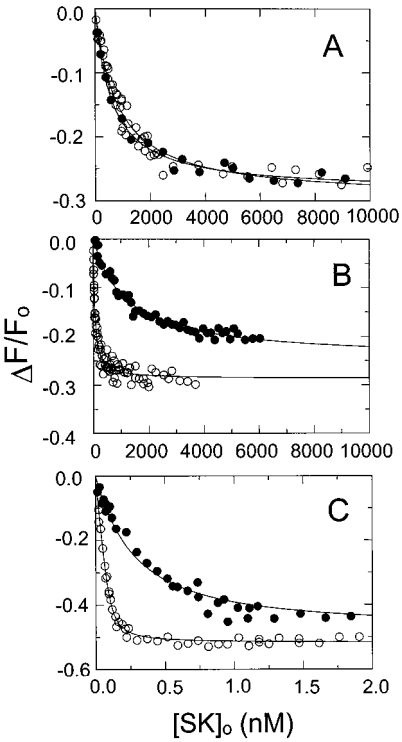

FIGURE 5: Effect of 6-AHA on SK binding to [5-F]FFR-labeled [Glu]Pg, [Lys]Pg, and [Lys]Pm at 125 mM Cl^-. The fractional changes in fluorescence ($\Delta F/F_o$) are plotted as a function of total concentration of SK ($[SK]_o$) for titrations in buffer containing 125 mM Cl^- of 30 nM [5-F]FFR-[Glu]Pg_1 (A), 15 nM [5-F]FFR-[Lys]-Pg_1 (B), and 75 pM [5-F]FFR-[Lys]Pm_1 (C) in the absence (○) and presence of 100 mM 6-AHA (●). Solid lines represent least-squares fits of the quadratic binding equation to the data with the parameters given in Table 1 and the text. Fluorescence titrations were performed and analyzed as described in Experimental Procedures.

investigate the involvement of lysine binding sites in the enhanced affinity of SK binding observed for the extended form of labeled [Glu]Pg at low Cl^- concentrations, the effect of 6-AHA on binding of SK to [5-F]FFR-labeled Pg and Pm was examined at 1 mM Cl^- (Figure 6). As was the case at 125 mM Cl^-, labeled [Lys]Pm_1 and [Lys]Pg_1 exhibited significant (11- and 28-fold, respectively) decreases in affinity for SK in the presence of saturating 6-AHA, indicating the involvement of lysine binding sites in these interactions that was largely independent of Cl^- concentration. In contrast to the absence of an effect of 6-AHA on [5-F]FFR-[Glu]Pg observed in 125 mM Cl^- (Figure 5), a

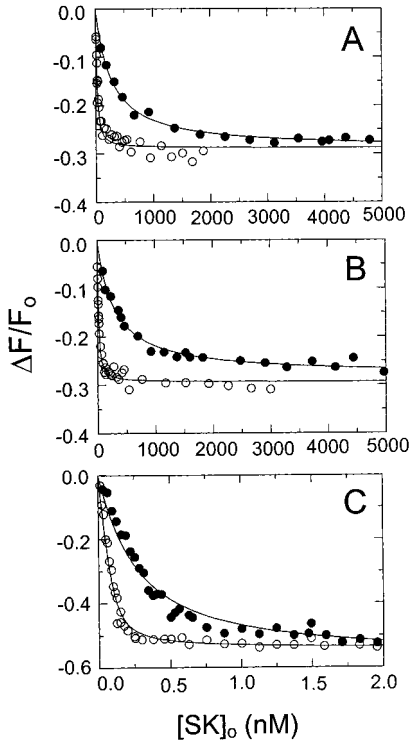

FIGURE 6: Effect of 6-AHA on SK binding to [5-F]FFR-[Glu]Pg, [5-F]FFR-[Lys]Pg, and [5-F]FFR-[Lys]Pm at low Cl^- concentrations. The fractional changes in fluorescence ($\Delta F/F_o$) are plotted as a function of total concentration of SK ($[SK]_o$) for titrations in buffer containing 1 mM Cl^- of 30 nM [5-F]FFR-[Glu]Pg_1 (A), 15 nM [5-F]FFR-[Lys]Pg_1 (B), and 75 pM [5-F]FFR-[Lys]Pm_1 (C) in the absence (○) and presence of 100 mM 6-AHA (●). Solid lines represent least-squares fits to the data with the parameters listed in Table 1 and the text. Fluorescence titrations were performed and analyzed as described in Experimental Procedures.

23-fold decrease in SK affinity for labeled [Glu]Pg was observed in the presence of 100 mM 6-AHA at 1 mM Cl^- (Figure 6 and Table 1), supporting the involvement of lysine binding site interactions.

Effect of Cl^- on Lysine Binding Site-Independent Interactions of SK with [5-F]FFR-Labeled Pg and Pm. To resolve the specific effect of Cl^- on Pg conformation from nonspecific ion effects, the lysine binding site-independent effects of Cl^- on binding of SK to [5-F]FFR-labeled Pm and Pg were examined in the presence of 100 mM 6-AHA (Table 1). The results demonstrated no detectable effect of low Cl^- concentrations on the affinity of SK for [5-F]FFR-[Lys]Pm_1 and modest 3.8- and 2-fold increases in the affinity of SK for labeled [Lys]Pg_1 and [Glu]Pg_1, respectively. These results demonstrated only a small increase in affinity at low Cl^- concentrations that was independent of lysine binding site interactions. To assess the contribution of lysine binding site interactions to the enhancement in the affinity of SK for [5-F]-FFR-[Glu]Pg at low Cl^- concentrations, the free energy change for this effect was compared to that for the change in affinity produced by 6-AHA at 125 mM Cl^-. After correction of the low Cl^- value for the small nonspecific ion effect, the free energy changes were indistinguishable, indicating that lysine binding site interactions could account for essentially all of the enhanced affinity for [5-F]FFR-[Glu]-Pg at low Cl^- concentrations.

DISCUSSION

The vastly different affinities of SK for [5-F]FFR-[Glu]-Pg, -[Lys]Pg, and -[Lys]Pm observed in the studies presented here at physiological salt concentrations demonstrate that conformational differences between labeled Pg and Pm species profoundly affect the interaction with SK and the mechanism of SK-induced fibrinolysis. SK binds with preferentially higher affinity to the extended conformations of fluorescein-labeled [Glu]Pg, [Lys]Pg, and [Lys]Pm in interactions facilitated by lysine binding sites, and to the active conformation of the [5-F]FFR-Pm catalytic domain. The combined result is a remarkable 41000−57000-fold difference in the affinity of SK for the labeled [Glu]Pg and [Lys]Pm analogues. The dissociation constants reported here for SK binding to labeled Pg and Pm species differ substantially from values reported previously by other investigators (*39*, *41*−*44*). A potentially significant methodological difference is the use of surface-immobilized proteins in those studies, whereas the studies presented here characterized the interactions in solution and under experimental conditions where proteolysis could be eliminated. The results of this study are in agreement with the dissociation constants of 11 ± 7 and 590 ± 110 nM reported for SK binding to analogues of [Lys]Pg_1 and [Glu]Pg_1, respectively, labeled at the catalytic site with the probe 2-anilinonaphthalene-6-sulfonic acid under slightly different experimental conditions (*40*), and with the affinity for native and labeled Pm obtained from kinetics and binding studies (*23*, *38*). The results contrast with those of other direct binding studies, however, particularly with respect to the very different affinities of SK for [Glu]Pg. Dissociation constants of 28 (*39*) and 420 pM (*44*) have been reported for SK binding to [Glu]Pg, which are 1400−21000-fold lower than the values determined here. The approximate agreement between the tighter dissociation constants and those determined here for [5-F]FFR-[Lys]Pm suggests that activation of Pg during previous studies may have been responsible for the apparently high affinity. It should also be recognized, however, that the dissociation constants for active site-labeled Pg and Pm derivatives are not expected to correspond exactly to those for binding of the native proteins. The presence of the probe−tripeptide label in the active site results in a 5−6-fold decrease in affinity for native [Glu]Pg (*40*), while no significant difference in affinity was found for labeled and native Pm (*23*). Although the dissociation constants may not be identical, the effects of the fluorescent label on SK affinity are relatively small and do not significantly alter the conclusions about the changes in affinity, the roles of lysine binding sites, and conformational changes. Last, it may be argued that the properties of labeled Pg were irreversibly altered by SK during active site labeling and dissociation of the SK−Pg complex through formation of an irreversibly activated free Pg species called the virgin enzyme (*50*). The formation of this species has only been reported under harsh denaturing conditions, however, and this is considered only a remote possibility to account for the relatively small effect of labeling on SK affinity for [Glu]Pg.

The results support a model for SK binding to Pg containing contributions from two relatively independent sources: a lysine binding site-independent interaction of SK with the catalytic domain and interactions of SK with lysine

binding sites on the kringle 4 and/or 5 domains of Pg. Although the affinities for labeled Pg and Pm were greatly different, the free energy benefit from the lysine binding site interactions was indistinguishable for [5-F]FFR-[Lys]Pg and -[Lys]Pm, indicating the relative independence of the contributions from the catalytic domain and lysine binding site interactions to the overall affinity. Like the effect of 6-AHA on SK binding, 100 mM benzamidine decreased the affinity of SK for [5-F]FFR-[Lys]Pg 15-fold, to a dissociation constant of 639 ± 113 nM,[2] suggesting that SK binding involves the lysine binding site of kringle 5, because this site is thought to be the only one that binds benzamidine (*32*, *51*). Binding of 6-AHA to kringles 4 and 5 has also been reported to be cooperative, however, which obviates the firm assignment of binding to a specific kringle (*52*). Although the crystal structure of SK bound to the Pm catalytic domain suggests that residues in the SK β-domain may interact with kringles in the analogous complex of SK with Pg (*22*), whether particular lysine residues of SK directly mediate this interaction, as is thought, is not clearly established. Previous studies of Pg binding to fibrin suggest that binding through lysine binding sites results in transition of [Glu]Pg to the more rapidly activated, extended conformation (*19*, *53*), similar to the interaction and conformational change observed here for SK and labeled Pg. The participation of lysine binding sites in SK binding to Pg and Pm, however, apparently does not interfere with the physiological binding of Pg and Pm to fibrin, as shown by previous studies demonstrating binding of the SK complexes (*54*).

The results shed new light on previous observations from kinetic studies of the SK activation mechanism. Stabilization of the compact conformation of fluorescein-labeled [Glu]-Pg by Cl^- binding resulted in a lower affinity for SK, which is correlated with its established relative resistance to activation by tissue-type plasminogen activator, urokinase, and SK at physiological levels of Cl^- (*33*−*35*). The results suggest that the previous observation that activation of [Glu]-Pg by SK was accelerated in the absence of Cl^- is likely due to a higher affinity of SK for the extended conformation of Pg as demonstrated here for the fluorescent analogue, which may contribute to the increase in k_{cat} observed for proteolytic activation of [Glu]Pg at low Cl^- concentrations (*34*). Increased reactivity of Pg in the extended conformation as a substrate of the SK−Pg complex may also play a role in the decrease in K_m for Pg observed at low Cl^- concentrations (*34*). The indistinguishable SK binding affinity and fluorescence changes observed for the fluorescein-labeled carbohydrate variants 1 and 2 of Pg and Pm showed that the N-linked carbohydrate chain at Asn^{289} that is present on form 1 and absent from form 2 is not involved in the SK interaction, and does not appear to affect the active site. This indicates that the reported differences in the SK-induced rate of activation of $[Glu]Pg_1$ and $[Glu]Pg_2$ are not due to differences in the affinity of SK for Pg, but may instead involve differences in the interaction of Pg as a substrate of the SK−Pg complex (*13*−*15*).

The 23-fold enhanced affinity of SK for the extended conformation of [5-F]FFR-[Glu]Pg suggests that [Glu]Pg "opens up" upon SK binding. SK may bind to the compact form of [Glu]Pg followed by rearrangement of Pg to the extended conformation due to the engagement of lysine binding sites. Whether the hypothetical compact complex is catalytically active is unknown. Alternatively, SK may bind to the extended conformation of [Glu]Pg in preexisting equilibrium with the compact form, forming an activated SK−Pg complex in which Pg is in an extended conformation. The idea that Pg in the SK−[Glu]Pg complex is in an extended conformation is compatible with earlier hydrodynamic studies which indicated that the conformation of this complex was not significantly affected by 6-AHA, in contrast to the effect on free [Glu]Pg (*55*). Binding of SK is the initiating event in the mechanism of [Glu]Pg activation, which is coupled closely to conformational activation of the Pg catalytic site, and subsequent proteolytic conversion of Pg to Pm. Recent studies of a recombinant SK mutant lacking the amino-terminal Ile residue demonstrated a critical role for this residue in the mechanism of conformational activation of Pg (*56*). Insertion of the SK amino terminus into the amino-terminal binding pocket of Pg has been concluded to be necessary to trigger conformational activation (*56*). Our results suggest that this critical event may be facilitated by initial formation of the SK−Pg complex and transition of Pg to the extended conformation as a possibly preceding step in the mechanism. Once Pg activation is initiated, the progressively higher affinities of [Lys]Pg and [Lys]Pm for SK are thought to drive the reaction to completion, with the ultimate formation of the most stable SK−Pm complex, which converts irreversibly the remaining free Pg to Pm.

[2] P. D. Boxrud and P. E. Bock, unpublished results.

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