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Analysis of the interactions between streptokinase domains and human plasminogen

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Abstract

The contrasting roles of streptokinase (SK) domains in binding human Glu1-plasminogen (Plg) have been studied using a set of proteolytic fragments, each of which encompasses one or more of SK's three structural domains (A, B, C). Direct binding experiments have been performed using gel filtration chromatography and surface plasmon resonance. The latter technique has allowed estimation of association and dissociation rate constants for interactions between Plg and intact SK or SK fragments. Each of the SK fragments that contains domain B (fragments A2-B-C, A2-B, B-C, and B) binds Plg with similar affinity, at a level approximately 100- to 1,000-fold lower than intact SK. Experiments using 10 mM 6-aminohexanoic acid or 50 mM benzamidine demonstrate that either of these two lysine analogues abolishes interaction of domain B with Plg. Isolated domain C does not show detectable binding to Plg. Moreover, the additional presence of domain C within other SK fragments (B-C and A2-B-C) does not alter significantly their affinities for Plg. In addition, Plg-binding by a noncovalent complex of two SK fragments that contains domains A and B is similar to that of domain B. By contrast, species containing domain B and both domains A and C (intact SK and the two-chain complex A1·A2-B-C) show a significantly higher affinity for Plg, which could not be completely inhibited by saturating amounts of 6-AHA. These results show that SK domain B interacts with Plg in a lysine-dependent manner and that although domains A and C do not appear independently to possess affinity for Plg, they function cooperatively to establish the additional interactions with Plg to form an efficient native-like Plg activator complex.

Keywords: fibrinolysis; lysine-binding site; plasminogen; serine protease; streptokinase; zymogen activation

Streptococcus equisimilis streptokinase (SK) is a single-chain secreted protein of 414 amino acids (Malke et al., 1985), which indirectly causes the activation of the blood plasma zymogen human plasminogen (Plg) to the fibrinolytic enzyme plasmin (Christensen, 1945) via active-site formation within Plg. This proceeds without proteolytic cleavage by means of a rapid and avid binding event followed by a slow and irreversible structural rearrangement within the complex, finally yielding a previously latent trypsin-like active center within Plg. The activator complex possesses specificity for the Arg561–Val562 peptide bond within the serine-protease domain of Plg substrate molecules, resulting in the generation of plasmin molecules (McClintock & Bell, 1971).

Understanding the manner by which SK coerces human Plg into forming an active site possessing Plg activator activity presents a considerable challenge. Each of these multidomain proteins, and each of their complexes, represents a system for which no high resolution structural information is currently available. From its sequence, however, Glu1-Plg is predicted to contain seven domains: an N-terminal peptide domain, five kringle domains, and a trypsinogen-like serine protease domain; these domains form intramolecular contacts that regulate the transitions between the three distinct conformations of Plg (Ponting et al., 1992a; Marshall et al., 1994). By contrast SK's three domains (A, B, and C) could not be predicted from its sequence; limited proteolysis and biophysical methods were therefore used for their delineation (Conejero-Lara et al., 1996; Parrado et al., 1996). SK·Plg complex formation

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Abbreviations: SK, *Streptococcus equisimilis* streptokinase (residues 1–414); Plg, human Glu1-plasminogen; NPGB, p-nitrophenyl p-guanidinobenzoate; 6-AHA, 6-aminohexanoic acid; SE-HPLC, size-exclusion high performance liquid chromatography; SPR, surface plasmon resonance; SK fragments: A1 (residues 1–63), B (residues 147–287 or 147–292), C (residues 288–380), B-C (residues 147–380), A2-B (residues 64–287 or 64–292), A2-B-C (residues 64–380).

appears to involve SK domains A and B (Reed et al., 1995; Rodríguez et al., 1995b; Young et al., 1995; Nihalani & Sahni, 1997) and the serine protease domain of Plg (Summaria & Robbins, 1976; Dawson et al., 1994; Lijnen et al., 1994).

Residues 145–290 have been shown to encompass SK's central domain B (Misselwitz et al., 1992; Conejero-Lara et al., 1996; Medved et al., 1996; Parrado et al., 1996), which participates in the binding of SK to Plg (Rodríguez et al., 1994, 1995b; Reed et al., 1995; Young et al., 1995; Nihalani et al., 1997) in a manner that may depend on a lysine-mediated interaction (Lin et al., 1996). However, this interaction alone is incapable of engendering Plg activation. The remaining Plg-binding determinants within SK have been proposed to reside within an N-terminal region (residues 1–90 approximately) (Shi et al., 1994; Rodríguez et al., 1995b; Young et al., 1995) contained within the N-terminal domain A (residues 1–145 approximately) (Conejero-Lara et al., 1996; Parrado et al., 1996). Additionally, the presence of domain C (residues 290–380 approximately) appears to be essential for active site formation in the SK·Plg complex and subsequently for its Plg activator activity (Reed et al., 1995; Young et al., 1995; Parrado et al., 1996).

This study makes use of a set of SK fragments that together represent over 90% of the SK polypeptide and which were obtained by limited chymotryptic proteolysis (Parrado et al., 1996). The interaction of these fragments with Plg at equilibrium in solution has been studied using gel filtration chromatography. Furthermore, surface plasmon resonance techniques (Schuck, 1997) have been used to investigate the kinetics of association and dissociation of these interactions. Results of these experiments further define the functions of the different structural regions of SK.

Results

Binding of SK fragments to plasminogen in solution monitored by gel filtration chromatography

The interaction between Plg and a variety of SK fragments in solution was assessed by gel filtration in 0.2 M phosphate buffer, pH 6.8, containing 10 mM 6-AHA. Control experiments using intact SK or Plg were performed to investigate the validity of the method to monitor Plg·SK complex formation (Fig. 1C). SK and Plg form a high-affinity 1:1 stoichiometric complex in an equimolar mixture of the two proteins as shown by the appearance of a single peak corresponding to the Plg·SK complex, which elutes earlier than both isolated Plg and SK. This high affinity binding event has been shown previously by gel filtration using similar conditions (Dawson et al., 1994).

Similar experiments were performed replacing SK with the following SK fragments: B (residues 147–292), C (288–380), A2-B (64–287 and 64–292), B-C (147–380), and A2-B-C (64–380). The results for fragments B-C and A2-B-C are shown in Figures 1A and 1B, respectively. Neither of these fragments show detectable binding to Plg under our experimental conditions, since the elution profiles of equimolar mixtures of the fragment with Plg contained two resolved peaks, which overlaid the peaks corresponding to the isolated moieties (Fig. 1A,B). Incubating the mixtures for 3 h at

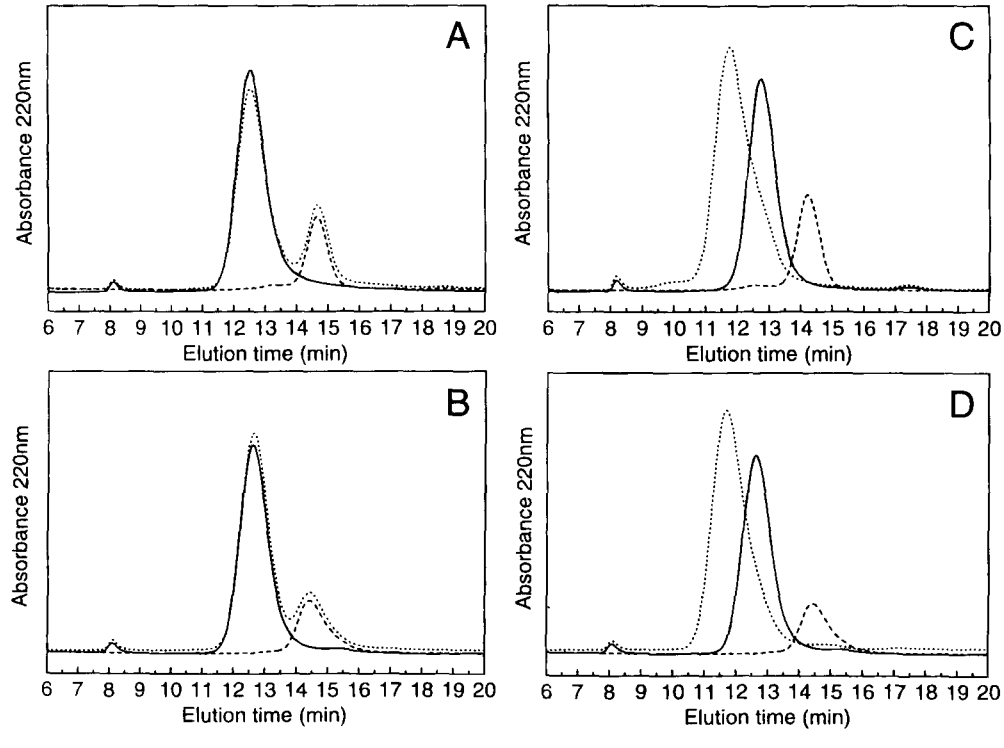

Fig. 1. Gel filtration elution profiles of mixtures of Glu1-Plg and either intact SK or SK fragments prepared as described in the text. Plots correspond to (**A**) fragments B-C, (**B**) A2-B-C, (**C**) intact SK, and (**D**) A2-B-C premixed with a fivefold molar excess of A1. The elution profiles shown are Plg control samples (solid line), isolated SK or SK fragments (dashed line), and equimolar mixtures of Plg with the different SK species (dotted line).

35 °C before chromatography or adding up to a 10-fold excess of the SK fragment did not result in the binding of either fragment to Plg (data not shown). These negative results are striking since several SK fragments, with sequences similar to the fragment B used here, have been reported to bind Plg with high affinities, approaching that of native SK (Reed et al., 1995; Rodríguez et al., 1995b; Young et al., 1995).

Consequently it was considered that the Plg-binding ligands 6-AHA and NPGB (Wohl, 1984; Thewes et al., 1990), present in these experiments, might inhibit the binding of SK fragments to Plg although it is notable that neither ligand was found to abolish the binding of intact SK to Plg. To investigate this possibility, fragment B-C was mixed with Plg in the absence of NPGB and was incubated for 30, 300, and 1,800 s prior to chromatography. Once more, no detectable complex formation occurred for these mixtures. Despite this, proteolytic degradation of the fragment was observed after 5 min of incubation and became extensive after 30 min of incubation, indicating a slow generation of plasmin. This observation is consistent with a very low but measurable Plg-activator activity of fragment B-C, reported previously (Rodríguez et al., 1995b; Parrado et al., 1996). Attempts to perform these gel filtration experiments in the absence of 6-AHA failed due to irreproducible and broad elution profiles of Plg as observed previously (Ling et al., 1967; Dawson et al., 1994).

The effect of SK fragment A1 (residues 1–63) on the binding of other SK fragments to Plg was investigated by the addition of A1 in a molar excess to SK fragments B-C, A2-B, and A2-B-C prior to mixing with Plg. The presence of a molar excess of fragment A1 in the mixtures of B-C or A2-B with Plg was found not to increase significantly complex formation (data not shown). For the latter case, the elution peak of SK fragment A2-B was observed to shift to longer elution times due to the presence of A1, without modifying the Plg elution profile. This observation is a consequence of the high affinity interaction of A1 with the region A2 of A2-B, with concomitant structural reconstitution of SK domain A, as recently demonstrated by gel filtration and nuclear magnetic resonance experiments (Conejero-Lara et al., 1996).

By contrast, addition of A1 to A2-B-C has a more dramatic influence on its binding to Plg (Fig. 1D). Whereas in the absence of A1, formation of the complex A2-B-C·Plg is not detectable, in the presence of a fivefold molar excess of A1 formation of the complex A1·A2-B-C·Plg is essentially complete. As with fragment A2-B, interaction of A1 with the region A2 of A2-B-C is known to reconstitute the native-like fold of SK domain A (Conejero-Lara et al., 1996). Therefore, reconstitution of domain A produces a dramatic increase in affinity of the noncovalent complex A1·A2-B-C for Plg. It is notable, however, that domain A reconstitution in the complex A1·A2-B, in the absence of domain C, does not affect its binding affinity for Plg.

Kinetics of the interactions of Plg with immobilized SK and SK fragments measured by surface plasmon resonance

The kinetics of association and dissociation of Plg with immobilized SK were measured by the BIAcore technique as described in Material and methods. Plg was injected at concentrations ranging from 5 nM to 1.5 μM. Figure 2A shows the results of an experiment performed with a sensor chip upon which SK was immobilized in an amount equivalent to 960 response units. For this experiment Plg samples were injected at concentrations ranging between 5 and 80 nM. Sensorgrams have been corrected for the bulk effect in the response signal arising from differences in the refractive indices of the buffer and the solution of analyte. A rapid and avid formation of the SK·Plg complex is seen to occur even at very low Plg concentrations (5–100 nM). Dissociation of the complex, however, is very slow due to the high stability of the SK·Plg complex.

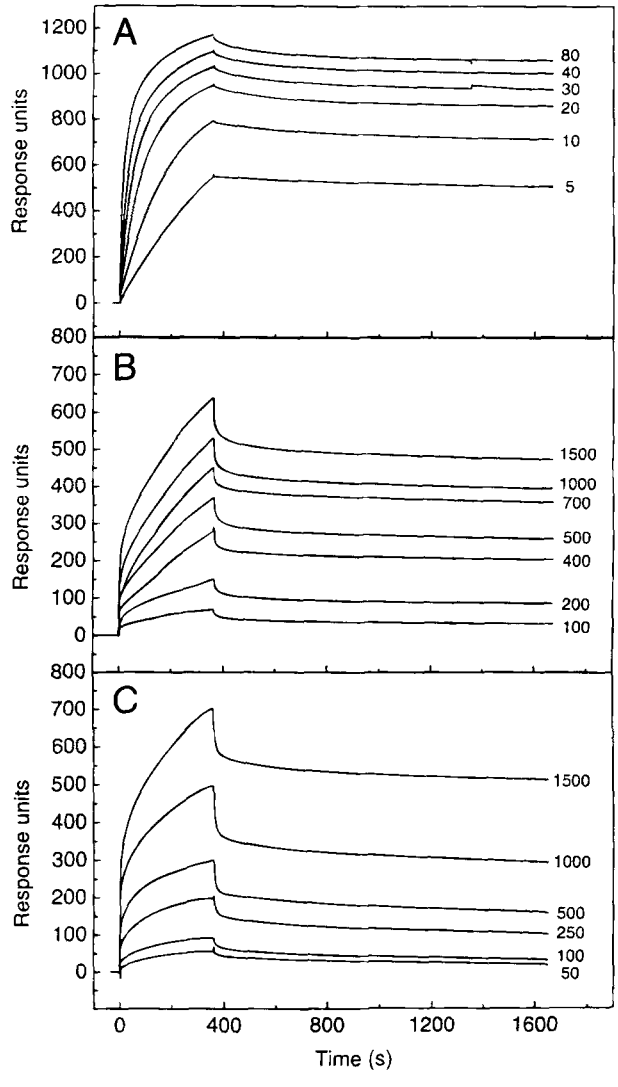

Fig. 2. SPR sensorgrams showing the binding of Plg to immobilized (**A**) SK, (**B**) fragment A2-B-C, and (**C**) fragment B-C. The SPR profiles represent the amount of bound Plg as a function of time during association and dissociation phases and for different analyte (Plg) concentrations, which are indicated in the plots in nanomolar units.

Experiments performed for SK or for SK fragments provide association and dissociation kinetics that differ considerably from monophasic behavior but are entirely consistent with biphasic characteristics. These characteristics are more pronounced at higher concentrations of Plg and are also observed as nonlinearities in $\ln(dR/dt)$ vs. time plots: such plots are linear for single-phase binding processes. SPR experiments using immobilized SK, A2-B-C, or B-C and Plg samples were performed using 5×10^{-5} M NPGB present in the injected Plg samples, in order to inhibit plasmin-mediated proteolysis. Under these conditions neither Plg nor SK is proteolysed by plasmin according to SDS-PAGE and gel

filtration experiments using SK-Plg complexes formed in the presence of NPGB. Experiments in the absence of NPGB with immobilized fragment B, which possesses negligible Plg activator activity, are well described by single association and dissociation events. Nevertheless, binding rate constants are similar to those observed for the binding of a majority of Plg molecules in the presence of NPGB (Table 1), indicating that 5×10^{-5} M NPGB does not significantly impair complex formation between domain B and Plg. These data suggest that the biphasic characteristics of experiments using NPGB result from a mixed population of Plg and Plg·NPGB molecules that differ in their SK-binding rate constants.

The curves representing the dissociation of Plg from immobilized SK are well described as the sum of two independent exponential decays. The fast phase accounts for approximately 3–6% of the amplitude, whereas the majority of the bound Plg molecules (94–97%) dissociate from SK during the slow phase with a rate constant k_d equal to $(1.7 \pm 0.9) \times 10^{-5}$ s^{-1}.

The kinetic data for the association of SK and Plg have been analyzed by global nonlinear least-squares fitting of a set of association curves obtained at different Plg concentrations, as described in Materials and methods. For intact SK, a global fitting procedure was adopted which fixed both dissociation rate constants $k_{d,1}$ and $k_{d,2}$ to the single slow phase rate constant that accounts for the majority of bound Plg molecules (see above). Thereafter, the fit was optimized by varying association rate constants $k_{a,1}$ and $k_{a,2}$, and the maximum responses for each binding event $R_{max,1}$ and $R_{max,2}$. Both the association and dissociation curves are well described by the model of two independent binding events (Fig. 3A; Table 1); relative amplitudes of the two association phases were found to be approximately 65 and 35%. Results for the binding of Glu1-Plg to SK indicate an avid interaction between these proteins, with apparent equilibrium dissociation constants in the subnanomolar range (Table 1).

Figures 2B and 2C represent SPR experiments that relate the binding of Plg to immobilized SK fragments B-C and A2-B-C. Plg was injected through the sensor chip at concentrations ranging between 50 and 1,500 nM. For each fragment the SPR response is highly Plg-concentration dependent and at Plg concentrations that are considerably greater than for the experiments using intact SK, indicating a much lower affinity of these SK fragments for Plg. Similar results were obtained for both immobilized fragments A2-B and B, the latter corresponding to the isolated central domain of SK (results not shown). No detectable binding of Plg to immobilized fragment C was observed in our experiments, even when injecting Plg at concentrations of up to 6 μM.

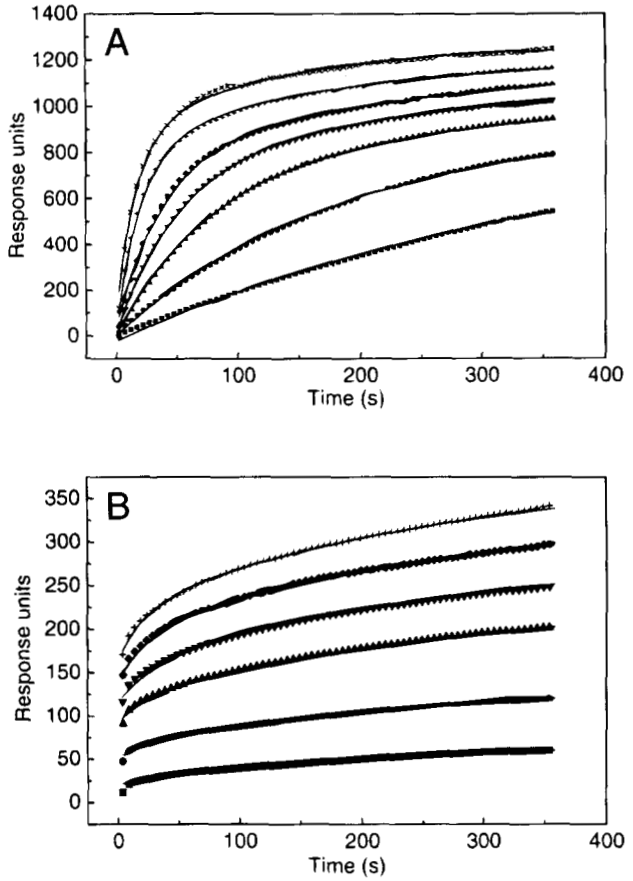

Fig. 3. SPR association curves with multiple nonlinear least-squares fits of the data superimposed. **A:** The analysis for the association of Plg (at concentrations of 5–100 nM) to immobilized SK. **B:** Corresponds to the association of Plg (at concentrations of 50–500 nM) to immobilized fragment B-C. Dots represent experimental data (sampled in intervals of 10 points for the sake of clarity) and lines correspond to the fits to the data.

Table 1. *Association and dissociation rate constants and apparent equilibrium dissociation constants for the binding of plasminogen to immobilized intact streptokinase and a variety of streptokinase fragments*[a]

	k_d ($\times 10^{-4}$) (s^{-1})	k_a ($\times 10^3$) ($M^{-1} \cdot s^{-1}$)	K_D ($\times 10^{-9}$) (M)
SK	0.17 ± 0.09	609 ± 60 (78 ± 12)	0.028 ± 0.019 (0.22 ± 0.16)
SK (+10 mM 6-AHA)	0.15 ± 0.07	72 ± 15 (15.1 ± 1.1)	0.21 ± 0.14 (1.0 ± 0.5)
SK (+50 mM benzamidine)	0.83 ± 0.13	181 ± 39 (21 ± 3)	0.46 ± 0.17 (3.9 ± 1.1)
A2-B-C	0.39 ± 0.04	3.6 ± 0.5	10.8 ± 2.6
B-C	1.19 ± 0.10	7.4 ± 0.5	16.1 ± 2.4
A2-B	1.3 ± 0.6	2.0 ± 0.3	65 ± 40
B	1.1 ± 0.6	1.0 ± 0.1	110 ± 71
B[b]	1.08 ± 0.15	2.7 ± 0.3	40 ± 10

[a] Values in parenthesis for intact SK correspond to the minor and slower association phase as described in the text.
[b] Measured in the absence of NPGB.

These results indicate that all fragments containing SK domain B form complexes with Plg, in accordance with previously reported results (Rodríguez et al., 1995b). Since 6-AHA was not used in these SPR experiments, the inability of SK domain B fragments to bind Plg in gel filtration experiments is likely to be due to competitive inhibition by 10 mM 6-AHA. This was confirmed using subsequent SPR experiments (see below).

As for SK, the dissociation and association kinetics of SK fragments that bind to Plg are biphasic. However, the dissociation rate constants of the SK fragments are substantially higher than those estimated for intact SK (Table 1). Most of the bound Plg (>90%) is found to dissociate in the slow phase, in a manner similar to intact SK, showing that these complexes, once formed, are similar to those containing intact SK in their kinetic stabilities. Analysis of the biphasic association curves was performed in a similar manner to that for intact SK (Fig. 3B). The only model that accurately described the data is of one independent binding event assigned to the most rapid dissociation and association phases, and a second event assigned the least rapid phases. The latter interaction dominates in these experiments as it corresponds to approximately 75–90% of the maximum SPR response amplitude. This slower binding event is considered to approximate the specific interaction of Plg with SK fragments in solution. Table 1 contains the rate constants and the apparent equilibrium constant calculated for the slower association and dissociation events corresponding to each of the SK fragments.

Despite the difficulties of quantitative data analysis due to the biphasic kinetics, the SPR results shown in Table 1 clearly indicate that all fragments that contain SK domain B form complexes with Plg and that these possess association and dissociation rate constants that are of approximately the same order of magnitude. These results strongly suggest that each of these SK fragments interacts predominantly with Plg via a binding site that is located within domain B. This agrees with previous reports that locate one of the binding sites of SK for Plg within the sequence regions 143–293 (Rodríguez et al., 1995b), 244–352 (Reed et al., 1995), and 234–293 (Nihalani et al., 1997). By contrast, full-chain SK shows much higher affinity for Plg, indicating the presence of additional binding determinants not present in any of the fragments studied here.

Effect of small molecule ligands on plasminogen binding to immobilized SK fragments

6-Aminohexanoic acid, a known ligand for Plg (Novokhatny et al., 1989; Thewes et al., 1990), was found to inhibit the interaction of Plg with SK and SK fragments in a concentration-dependent manner. The initial rate of Plg binding to different immobilized SK species was measured as a function of 6-AHA concentration. Figure 4 shows initial binding rates versus 6-AHA concentration calculated from SPR experiments as described in Materials and methods. Analysis of these data provides an estimate of the apparent equilibrium dissociation constants of the Plg·6-AHA complex. The results are shown in Table 2 for each SK species.

The 10 mM 6-AHA was found to inhibit completely the binding of Plg to each of immobilized SK fragments B-C, A2-B, and B. These results are entirely consistent with the gel filtration findings and support our previous conclusion that each of these SK fragments interacts with Plg exclusively via a single site located within domain B. For intact SK, however, 10 mM 6-AHA reduces the association rate and binding affinity for Plg to levels that remain high in comparison with the fragment data (see Table 1). Apparent Plg·6-AHA equilibrium dissociation constants are largely independent of the SK fragment used (Table 2), which indicates that, within Plg, 6-AHA competes for the same site of interaction as each of the SK fragments and intact SK. This implies that the 6-AHA-inhibitable interaction occurs via SK domain B.

Further experiments were carried out to monitor the effect of benzamidine on SK(fragment)·Plg complex formation. Fifty millimolar benzamidine was found to abolish the binding of Plg to fragments B and B-C. By contrast, the presence of 50 mM benzamidine only reduces the binding affinity of SK for Plg in a similar manner to that of the 10 mM 6-AHA experiments (Table 1).

Interactions between different streptokinase fragments measured by surface plasmon resonance

Surface plasmon resonance has also been used to measure rate and equilibrium binding constants describing interactions between the N-terminal fragment A1 (residues 1–63) and fragments A2-B (64–287), A2-B-C (64–380), and B-C (147–380). Fragment A1 was immobilized on the sensor chip surface as described in Materials and methods, and samples of SK fragments were injected at 20 μL/min during an association phase of 8 min and at concentrations ranging from 50 to 500 nM for fragments A2-B and A2-B-C, and from 200 nM to 2 μM for B-C. Dissociation phases of 25 min were recorded following injection (Fig. 5). In these experiments the sensor surface was regenerated after each cycle using 6 M GuHCl.

Binding of fragments A2-B and A2-B-C to A1 has been shown previously by gel filtration and NMR to reflect the interaction between two complementary portions of SK domain A, resulting in reconstitution of its native structure (Conejero-Lara et al., 1996). Table 3 shows the rate and equilibrium constants obtained for these interactions estimated by SPR. Similar dissociation constants were found for the binding of fragments A2-B and A2-B-C to immobilized A1. Unexpectedly fragment B-C, which lacks domain A, also has a measurable interaction with immobilized A1 (Fig. 4C). The association constant found for this interaction is lower than that for fragments A2-B and A2-B-C (see Table 3), whereas dissociation rate constants for all the three fragments are similar. The low-affinity interaction between fragments B-C and A1 was not detected by gel filtration or by NMR (Conejero-Lara et al., 1996). This interaction between A1 and B-C might explain the known potentiation effect that A1 has on the Plg-activator activity of B-C (Parrado et al., 1996).

Effect of reconstitution of SK domain A in interactions with Plg

These and other experiments (Conejero-Lara et al., 1996) show that SK domain A is able to be structurally reconstituted from unstructured fragments A1 (1–63) and A2 (64–146). In order to monitor the effect of this on the interactions between SK fragments with Plg, experiments were again performed using A1 immobilized on the sensor surface. The double injection facility of the BIAcore 2000 instrument was used to inject 2 μM B-C, or 500 nM A2-B-C, or 500 nM A2-B for 5 min, followed immediately by a 6 min injection of Plg at concentrations ranging from 10 nM to 1 μM (Fig. 6). As shown previously, each of these three fragments forms a complex with immobilized A1 during the initial stage of the experiment. During the Plg injection stage, the pre-formed two-fragment complexes A1·A2-B and A1·B-C each respond in a

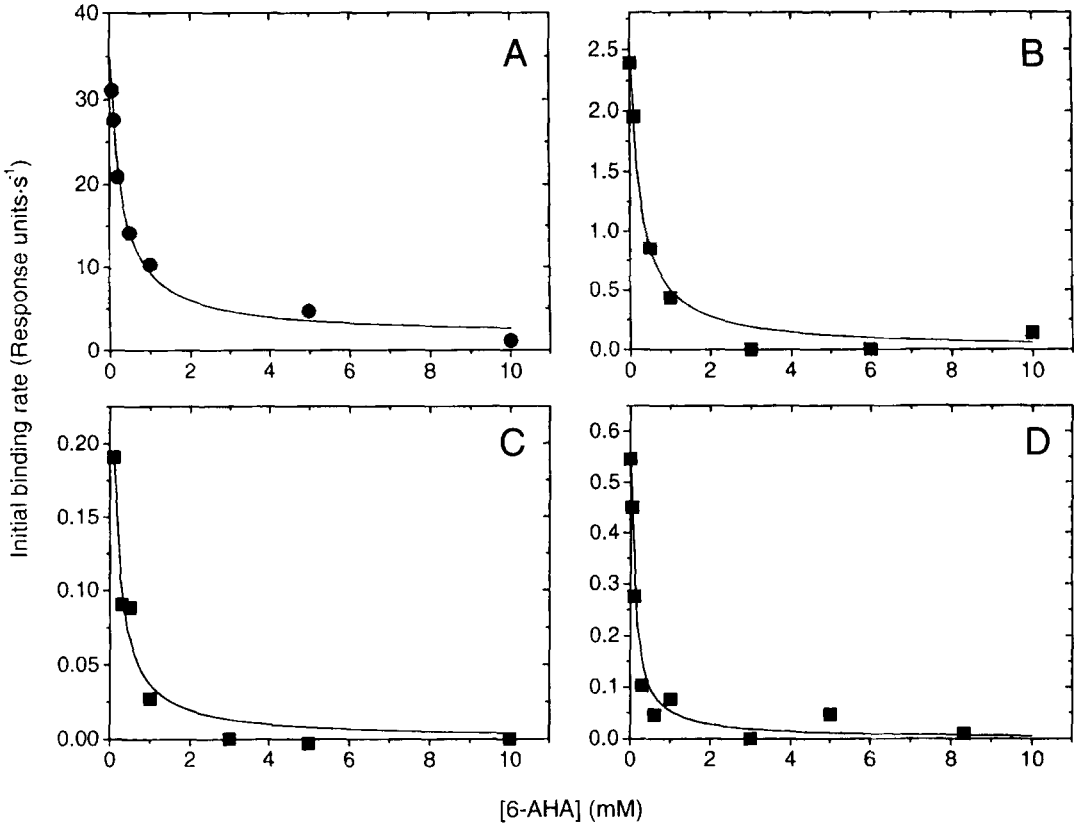

Fig. 4. Plots of initial rate of Plg binding r_o versus 6-AHA concentration for either immobilized SK or SK fragments measured by SPR. Dots correspond to the experimental values obtained for association of Plg to (**A**) intact SK, (**B**) B-C, (**C**) A2-B, and (**D**) B. The fits of Equation 3 to the data using a nonlinear least-squares procedure are shown in solid lines. Fits corresponding to the SK fragments were performed using a value of zero for the apparent rate constant k_2, as described in Materials and methods. Calculated values for the equilibrium dissociation constant (K_D) of Plg·6-AHA are listed in Table 2.

manner similar to the injection of Plg over immobilized fragment B. Due to the presence of signal artifacts produced by the double injection system of the instrument (see Fig. 6) and the concurrent dissociation of A1 from A1·A2-B or A1·B-C complexes, quantitative analysis of these curves was not attempted. However, the Plg concentrations necessary to produce a significant level of binding indicate that the observed affinities of A1·A2-B and A1·B-C for Plg are similar to that already observed for domain B. This indicates that interaction of fragment A1 with either of these two SK fragments does not promote an increase in their Plg binding capability.

Interaction of Plg with the two-chain complex A1·A2-B-C reveals a different result (Fig. 6). Binding of Plg is rapid and avid even at very low Plg concentrations, and is similar to that found for intact SK. It is apparent that, in the presence of domain C, reconstitution of domain A promotes a dramatic increase in the affinity of SK fragments for Plg, in agreement with the gel filtration data described above.

Table 2. *Apparent dissociation constants for the binding of 6-AHA to Plg calculated from 6-AHA-mediated inhibition on the rates of association of Plg to different immobilized SK moieties*

	K_D(Plg·6-AHA) ($\times 10^{-3}$) (M)
SK	0.29 ± 0.06
B-C	0.24 ± 0.05
A2-B	0.11 ± 0.05
B	0.10 ± 0.02

Table 3. *Association and dissociation rate constants and equilibrium dissociation constants for the interaction between immobilized fragment A1 and three different streptokinase fragments*

	k_d ($\times 10^{-3}$) (s^{-1})	k_a ($\times 10^{3}$) ($M^{-1}\cdot s^{-1}$)	K_D ($\times 10^{-6}$) (M)
A2-B	1.03 ± 0.03	6.1 ± 0.2	0.17 ± 0.01
A2-B-C	0.99 ± 0.02	3.9 ± 1.6	0.25 ± 0.11
B-C	1.11 ± 0.04	1.08 ± 0.04	1.03 ± 0.07

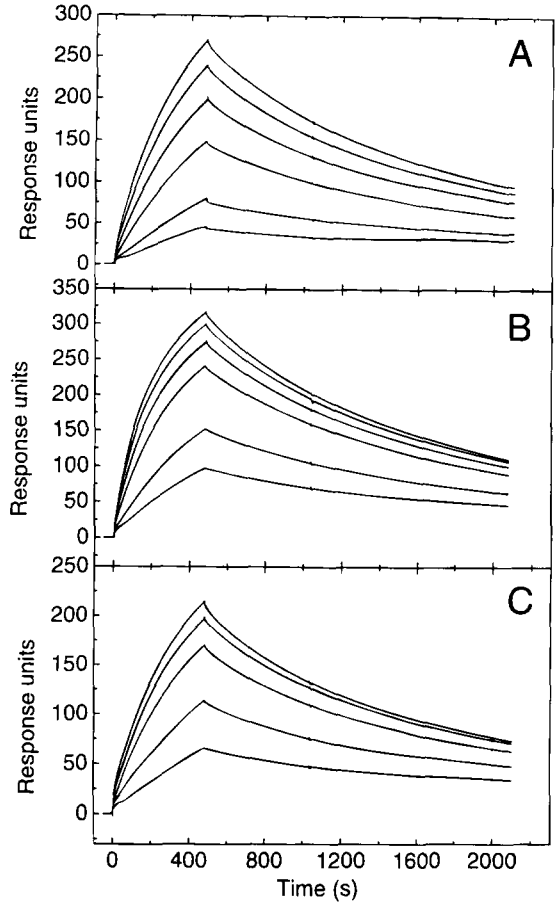

Fig. 5. SPR sensorgrams showing the interaction of SK fragments (**A**) A2-B-C, (**B**) A2-B, and (**C**) B-C with immobilized fragment A1. Fragment A1 was immobilized on the sensor surface and SK fragment solutions were injected for 8 min followed by injection of buffer alone. Curves with increasing responses correspond to SK fragments concentrations of 50, 100, 200, 300, 400, and 500 nM for **A** and **B**, and 200, 500, 1,000, 1,500, and 2,000 nM for **C**.

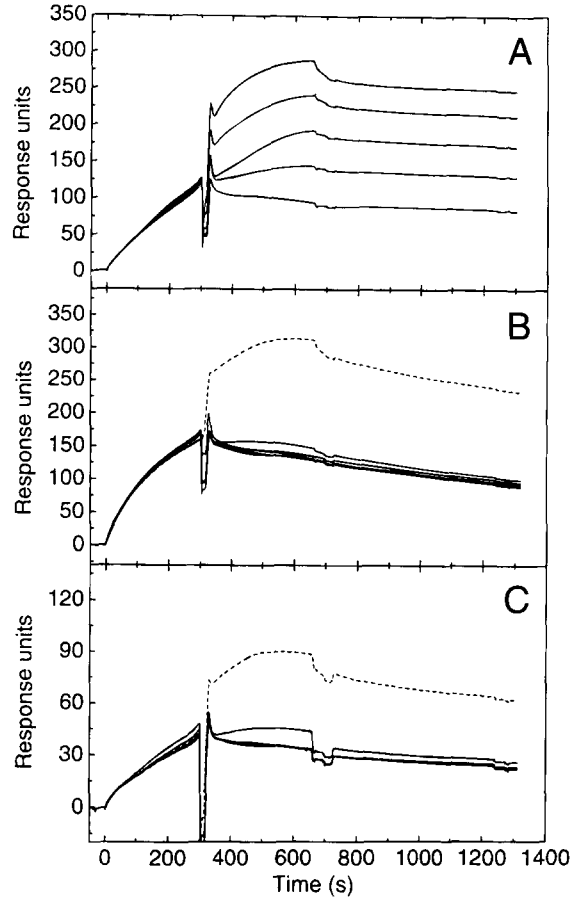

Fig. 6. SPR sensorgrams showing the binding of Plg to two-chain complexes of SK fragments pre-formed on the sensor surface as shown in Figure 5. Fragment A1 was immobilized on the sensor surface and the SK fragments were injected during 5 min at concentrations of 500 nM for (**A**) A2-B-C and (**B**) A2-B and 2 μM for (**C**) B-C. Immediately following SK complex formation, Plg was injected for a period of 6 min at different concentrations: 0 (control), 10, 30, 50, and 100 nM (shown as solid lines). For fragments A2-B and B-C, an additional experiment using 1 μM Plg was performed (**B** and **C**, dashed line). The 6 M guanidine hydrochloride was finally injected after each cycle to regenerate the sensor surface. Artifacts separating the two injection stages result from imperfections during consecutive injections.

Discussion

Size-exclusion HPLC (SE-HPLC) and surface plasmon resonance (SPR) experiments have been used to investigate potential interactions between the three domains of SK (A, B, and C) and full-length human Glu1-Plg. Despite complications arising from the necessity of using the plasmin inhibitor NPGB in SPR experiments and also 6-AHA in SE-HPLC experiments, the present results clearly show that all SK fragments that contain the central B domain (namely, A2-B-C, B-C, A2-B, and B) are capable of forming complexes with Glu1-Plg; these findings are in accord with previous studies (Rodríguez et al., 1994, 1995b; Reed et al., 1995; Young et al., 1995; Nihalani et al., 1997). The association and dissociation kinetic constants reported here for the binding of Plg to a variety of SK fragments are similar in magnitude indicating that all the fragments interact with Plg essentially via a single binding site located within domain B. By contrast, SK forms a complex with Plg two orders of magnitude more rapidly than any of the isolated SK fragments, while the dissociation rate constants of SK(fragment)·Plg complexes are approximately one order of magnitude higher when compared with that of intact SK·Plg. Consequently, the overall apparent Plg-affinity for each of these isolated fragments is reduced by a factor of more than 100 when compared with that of intact SK (Table 1). These results indicate that full-length SK contains additional determinants for the interaction with Plg that are not present within these fragments.

Gel filtration and SPR experiments show that the binding of SK domain B to Plg is essentially abolished by the presence of saturating concentrations of either 6-AHA or benzamidine. Addition of these known Plg-binding ligands (Novokhatny et al., 1989; Thewes et al., 1990) also markedly reduces the binding of intact SK to Plg, presumably due to inhibition of domain B mediated interaction(s) with Plg. Nevertheless, intact SK retains a high affinity for Plg in the presence of either 10 mM 6-AHA or 50 mM benzamidine, indicating that the additional SK·Plg binding determinants are insensitive to each of these ligands. Bock et al. (1996) recently showed evidence that 6-AHA reduces the affinity of intact SK for

Plg. These results appear to clarify a report from Lin et al. (1996) that a SK variant, containing alanine substitutions of lysine residues 256 and 257 located within domain B, shows a 63% decrease in Glu1-Plg–binding affinity.

The simplest model that accounts for these data is that SK Lys256 and/or Lys257 compete with lysine analogues 6-AHA or benzamidine for a binding site within Plg. Evidence suggests that this SK-binding site in Plg is the lysine-binding site of kringle 5. It is noted that apparent dissociation constant values for Plg·6-AHA estimated from our experiments of binding inhibition are in the range of 100–300 μM. These values are greater than dissociation constants known for 6-AHA–binding sites located within Plg kringles 1 and 4 (Lerch et al., 1980) yet are similar to that known for isolated kringle 5, $K_D = 95$ μM (Thewes et al., 1990). Significantly, kringle 5 is the only Plg kringle domain that possesses significant affinity for benzamidine (Váradi & Patthy, 1981; Thewes et al., 1990) although this compound can also bind the cryptic active center of Plg (Wohl, 1984).

The suggestion that SK domain B binds to the Plg kringle 5 lysine-binding site implies that SK competes with the internal ligand within the Plg NTP for this site (Ponting et al., 1992a; Marshall et al., 1994). It is perhaps significant, therefore, that (1) SK binds Lys-Plg (Plg that lacks the NTP) with considerably higher affinity than Glu1-Plg (Bock et al., 1996), and (2) that, unlike Glu1-Plg, SK·Glu1-Plg appears not to alter its conformation upon the addition of saturating concentrations of 6-AHA (Barlow et al., 1984). It is plausible, therefore, that the binding of SK to Glu1-Plg induces a conformational change in the latter molecule resulting in a more extended structure. Indeed, if this binding event occurs when the SK molecule is integral to the SK·Plg activator complex, and Glu1-Plg is a substrate molecule, then Plg activation is likely to occur more rapidly as a direct result of the greater accessibility of the Glu1-Plg activation peptide bond to activators in its extended conformation (reviewed in Ponting et al., 1992b; Markus, 1996). The binding of SK domain B to Plg kringle 5 might also account for its enhancement of staphylokinase·Plg complex formation and active site exposure (Rodríguez et al., 1995a) given that the staphylokinase-binding site on Plg is thought to reside within its serine protease domain, and that this site is likely to be inaccessible within the compact conformation of unliganded Glu1-Plg.

Isolated SK domain C (residues 288–380) does not interact significantly with Glu1-Plg. This result correlates with the inability of this fragment to induce Plg activator activity despite its native-like structure (Parrado et al., 1996). By contrast, fragments containing domains B and C (fragments A2-B-C and B-C) demonstrate Plg-affinities similar to that of domain B, which are abolished by saturating 6-AHA concentrations. Similar results were obtained for fragment A1·A2-B. Although at present we cannot exclude the possibility that intact single chain domain A binds Plg, we conclude that high-affinity interactions of fragments A1·A2-B, A2-B-C, or B-C with Plg are mediated solely by the Plg-binding site within domain B. This contrasts with a previous finding that a sequence similar to A1 binds Plg (Nihalani & Sahni, 1997).

Samples containing all three SK domains, namely the A1·A2-B-C complex or intact SK, demonstrate dramatic increases in Plg-affinity in comparison to those of smaller fragments. The high affinities of the three-domain molecules are relatively insensitive to disruption of the domain B mediated interaction by the addition of 10 mM 6-AHA. It appears that, whereas domains A and C do not possess significant Plg-binding function in isolation, these domains function cooperatively in forming high-affinity Plg-binding sites.

Other studies have indicated the existence of interdomain cooperativity during SK-mediated Plg activation. Domains B- and C-containing fragments are known to be the minimal SK sequences capable of generating low but measurable Plg activator activity (Rodríguez et al., 1995b; Parrado et al., 1996). Although amidolytically active, these fragments possess reduced plasmin-generating capabilities (Young et al., 1995). Similarly, a SK variant with domain C lysines 332 and 334 substituted with alanines, possesses native SK-like abilities both to form a Plg·SK complex and to bind a substrate Plg molecule, yet possesses a reduced catalytic rate constant for Plg activation (Lin et al., 1996). It appears therefore that whereas SK domain B mediates an interaction with Plg within the activator complex (this study; Shi et al., 1994; Young et al., 1995; Parrado et al., 1996), the function of inducing the active SK·Plg complex to proteolyse specifically the activation peptide bond of substrate Plg molecules appears to reside within domain C (Young et al., 1995).

In conclusion, each of the three SK domains is involved, either directly or indirectly, in the interaction of SK with Plg. Binding of SK to Plg mediated by domain B is abolished by lysine analogues, although this does not greatly reduce the affinity of native SK for Plg. Domain B binding to Plg is proposed to involve the latter's lysine binding site within kringle 5, which would result in Glu1-Plg adopting a elongated conformation similar to that of native Lys-Plg (Marshall et al., 1994). Both domains A and C cooperate to establish additional high affinity, nonlysine dependent, interactions with Plg. These two domains are thus necessary but not sufficient for native-like Plg·SK complex formation. The three domains of SK therefore function in concert by establishing a SK·Plg activator complex, inducing its hydrolytic activity, and altering its substrate specificity.

Materials and methods

Proteins

Purified streptokinase from culture filtrates of *Streptococcus equisimilis* was supplied by SmithKline Beecham Pharmaceuticals (Gronau, Germany). Purity of the protein was assessed by SDS-PAGE to be greater than 95%. Streptokinase fragments were obtained by chymotryptic cleavage of the intact protein and purified to homogeneity as described previously (Parrado et al., 1996); samples were stored frozen at −20 °C. Human Glu1-plasminogen (<2% Lys-plasminogen) was purchased from Biopool (Umeå, Sweden). p-Nitrophenyl p-guanidinobenzoate (NPGB), benzamidine, and 6-aminohexanoic acid (6-AHA) were purchased from Sigma Chemical Co. (Poole, UK). Remaining chemicals were of analytical grade.

Prior to experimentation, protein samples were buffer-exchanged using Sephadex G-25 PD-10 columns (Pharmacia, Uppsala, Sweden). Protein concentrations were estimated from absorption measurements at 280 nm, using absorption coefficients $\epsilon^{0.1\%}$(280 nm) for SK and SK fragments estimated from their amino acid sequence (Parrado et al., 1996) and $\epsilon^{0.1\%}$(280 nm) = 1.68 for Glu1-plasminogen (Wallén & Wiman, 1972).

Gel filtration chromatography

Binding of SK and SK fragments to Plg was monitored by gel filtration, using a 1 cm × 30 cm Superose-12 column (Pharmacia,

Uppsala, Sweden) attached to a Gilson HPLC instrument equipped with an automated sample injector. The column was equilibrated at room temperature in a 10 mM 6-AHA, 0.2 M phosphate buffer, pH 6.8; 6-AHA is necessary for the elution of Plg and SK-Plg complexes from the column (Ling et al., 1967; Dawson et al., 1994). Samples of 100 μL were injected using a flow rate of 0.8 mL/min. In a typical experiment, complex formation was assessed by mixing Plg and either SK or SK fragments at different molar ratios, keeping the final Plg concentration constant at 1 μM. A 50-fold molar excess of the inhibitor NPGB (Chase & Shaw, 1969) was present in the mixtures in order to prevent plasmin-mediated degradation of proteins. SDS-polyacrylamide gel electrophoresis of the mixtures showed no evidence for plasmin activity in the samples. This was also confirmed by the absence of proteolytic degradation of the proteins in the mixtures, even after long incubation periods, despite the high sensitivity of intact SK and some of the SK fragments to proteolytic degradation. Additional control elution experiments were performed with isolated Plg, SK, and SK fragment samples. Elution profiles were recorded by monitoring absorbance at 220 nm.

Surface plasmon resonance

Association and dissociation between Plg and the different SK moieties were followed in real-time using a BIAcore 2000 instrument (Pharmacia, Uppsala, Sweden) based on the process of surface plasmon resonance (SPR). Either SK or a SK fragment was immobilized on the surface of a sensor chip (type CM5, containing a carboxymethylated dextran matrix) using the amine coupling kit, as recommended by the manufacturer. Immobilization was performed using protein solutions in 10 mM acetate, pH 5.0 at a flow rate of 5 μL/min over a period of 6 min. Different levels of immobilization (200 to 3,000 response units) were regulated by injection of protein solutions at concentrations between 0.01 and 0.1 mg/mL.

Plg samples were injected over the sensor surface at 20 μL/min in 0.2 M phosphate buffer, 0.005% surfactant P20, pH 6.8. In general, all injected Plg samples contained 5×10^{-5} M NPGB to prevent plasmin-mediated degradation of the immobilized protein as with the gel filtration experiments. A variety of Plg concentrations was used for each SK moiety (ranging from 5 nM to 2 μM, dependent on the apparent levels of binding). In a typical experiment, association phases were recorded during a period of 6 min following Plg injection. Dissociation phases were recorded for 20 min by washing with buffer alone. Following each cycle, bound Plg was released from the sensor surface by injection of solutions of guanidine hydrochloride, the concentration of which was optimized between 1.5 M and 6 M for each different experiment in order to obtain the optimum reproducibility of results.

SPR binding experiments that immobilized Plg, rather than SK, to the chip surface were also initiated. These were, however, unsuccessful since the SPR response signal observed for the binding of SK to immobilized Plg was irreproducible following the first injection of SK. This phenomenon persisted even upon the addition of 5×10^{-5} M NPGB in an attempt to arrest plasmin-mediated proteolysis.

Data analysis was performed by nonlinear least-squares analysis as described by O'Shannessy et al. (1993) using the Levenberg–Marquardt algorithm as implemented in the Origin 4.1 software (Microcal Software, Northampton, Massachusetts). Association and dissociation kinetics were both observed to be biphasic for the majority of the SK species studied. The simplest scheme that can account for the SPR sensograms consists of two independent interactions (see Results). Under this assumption, the SPR response signal is considered to be the sum of signals arising from two independent binding events $R_1 + R_2$.

Dissociation and association SPR curves were fitted using the equations reported by O'Shannessy et al. (1993). Dissociation curves were first fitted in order to obtain values for the two dissociation rate constants $k_{d,1}$ and $k_{d,2}$ for each concentration of analyte (Plg). Values of the kinetic constants for each analyte concentration were then averaged for use in a subsequent estimation of association rate constants $k_{a,1}$ and $k_{a,2}$ achieved by fitting the association curves. For this, the complete set of association curves obtained at different analyte concentrations was fitted simultaneously using a global fitting procedure, in which a single set of parameters was used and each curve related to the equations by the value of the analyte concentration. This approach was adopted because fitting to single association curves was found to be unreliable as the association curves for lower Plg concentrations provided insufficient information to describe accurately the slow binding events. In addition, curves at higher Plg concentrations were unable to describe accurately the fast binding events. Fitting was constrained by values of the dissociation rate constants estimated from analysis of dissociation curves. To determine which of the two calculated dissociation constants $k_{d,1}$ and $k_{d,2}$ was pertinent to each of the association processes, the three possible combinations of the two k_d values were used as constraints in different fitting attempts. The two combinations of k_d values that produced best fits to the data were taken to represent the two independent binding interactions. Parameter confidence intervals were estimated from the χ^2-minimization algorithm as provided by the Origin software. For each of the two binding events, a value of the apparent equilibrium dissociation constant (K_D) was calculated as k_d/k_a. The theoretical SPR responses for saturation with Plg could not be measured directly in these experiments at reasonable injection times and sample concentrations as a result of the slow association and dissociation rates observed for all SK fragments. They were, however, obtained from the nonlinear least-squares analysis of the association curves.

The effects of known Plg ligands, 6-AHA and benzamidine (Novokhatny et al., 1989; Thewes et al., 1990), on the binding of Plg to the SK immobilized moieties were assessed by measuring the initial association rates of Plg in the presence of different ligand concentrations at a constant Plg concentration. For the analysis of these data, it was assumed that effector-bound Plg has a reduced association rate constant for intact SK, whereas only Plg molecules free of effector can bind the SK fragments (see Results). It follows from these approximations that, in general, for each concentration of inhibitor $[I]$, the initial rates for the binding of Plg to immobilized SK moieties are

$$r_o = k_1 [\text{Plg}] + k_2 [\text{Plg}\cdot I] \quad (1)$$

where k_1 and k_2 are apparent rate constants that are proportional to the amount of immobilized SK molecules and to the association rate constants of free Plg and effector-bound Plg, respectively. The apparent dissociation constant K_I for the binding of the inhibitor I (6-AHA or benzamidine) to Plg is given by

$$K_I = [\text{Plg}][I]_o/[\text{Plg}\cdot I] \quad (2)$$

where, in the presence of an excess of I, the concentration of free inhibitor $[I]$ can be considered approximately equal to the total concentration of inhibitor $[I]_o$.

Taking into account that the total concentration of Plg is $[Plg]_o = [Plg] + [Plg \cdot I]$, it follows that

$$r_o = k_1 K_I [Plg]_o / (K_I + [I]_o) + k_2 [I]_o [Plg]_o / (K_I + [I]_o). \quad (3)$$

This equation relates the initial Plg association rates to the concentration of inhibitor $[I]_o$. Initial binding rates r_o were estimated for each inhibitor concentration from the derivatives of association curves at times subsequent to the termination of bulk effects in the SPR response signals, for which the signal represented specific protein–protein interactions. Values of the initial binding rates obtained at different times of association give consistent results in this analysis. Values of r_o as a function of the total concentration of inhibitor $[I]_o$ were fitted by nonlinear least-squares methods using Equation 3, which allowed estimation of values for k_1, k_2, and K_I. For the analysis of data from the SK fragments, the value of k_2 was fixed to zero since both effectors completely inhibit the interaction of SK fragments with Plg (see Results). For intact SK the value obtained for k_2 was approximately one order of magnitude lower than that for k_1, in agreement with values obtained for the Plg association rate constants for intact SK in the absence and presence of the effectors (see Results).

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