Substitution of Arginine 719 for Glutamic Acid in Human Plasminogen Substantially Reduces Its Affinity for Streptokinase†

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ABSTRACT: In isolation human plasminogen possesses no enzymatic activity, yet upon formation of an equimolar complex with the bacterial protein streptokinase, it acquires a plasminogen activator function. The region(s) of plasminogen and of streptokinase which mediate complex formation has (have) not been previously published. Here it is reported that a single-residue substitution ($Arg_{719} \rightarrow Glu$) in the serine protease domain of full-length Glu-plasminogen substantially reduces its affinity for streptokinase. The plasminogen variant displays no other significant differences from the wild-type molecule with respect to activation by two-chain urokinase-type plasminogen activator, recognition by monoclonal antibodies, or ability to undergo conformational change. It is concluded that Arg_{719} in human plasminogen is an important determinant of the streptokinase binding site, although further sites are likely to contribute both to the affinity of plasminogen for streptokinase and to mechanisms by which the active site is formed within the complex.

The serine protease plasmin is generated from its inactive precursor plasminogen by proteolytic cleavage of the Arg_{561}–Val_{562} peptide bond. This reaction is catalyzed by tissue-type or urokinase-type plasminogen activators (t-PA, u-PA)[1] or by a noncovalent stoichiometric complex of plasmin(ogen) with streptokinase (Christman *et al.*, 1977; McClintock & Bell, 1971). Plasmin, therefore, consists of two chains: an A-chain containing an N-terminal peptide and five kringle domains and a B-chain serine protease domain (Sottrup-Jensen *et al.*, 1978). The protease domain possesses an active site which is able to hydrolyze peptide bonds within (*inter alia*) fibrin, single-chain plasminogen activators (t-PA and u-PA), and its inhibitor, α_2-antiplasmin, with which it forms an essentially irreversible complex (Wiman & Collen, 1978; Rijken & Collen, 1981; Nielsen *et al.*, 1982). It is, however, unable to activate plasminogen except in the presence of either streptokinase or staphylokinase, another bacterial nonenzymatic activator (Collen *et al.*, 1993).

Streptokinase (SK) is a single-chain, monomeric and secreted protein (molecular weight ≈47 000) produced by several strains of β-hemolytic streptococci. In isolation SK has never been shown to possess enzymatic activity. It is thought that an active site is formed within the plasminogen serine protease domain upon formation of the streptokinase–plasminogen complex. This active site differs from that of plasmin since it possesses plasminogen activation activity and is inhibited by α_2-antiplasmin at a rate 2×10^7-fold slower than plasmin (Cederholm-Williams *et al.*, 1979; Wiman, 1980). It is assumed that the effect of complex formation is to generate an oxyanion hole, similar to that generated during the activation of zymogen serine proteases (Huber & Bode, 1978). The plasminogen activation ability of the complex is exploited in the use of streptokinase as an efficacious agent in the clinical treatment of acute myocardial infarction caused by coronary thrombosis (ISIS-3, 1992).

Plasminogens isolated from different mammalian species exhibit variation in their response to *Streptococcus equisimilis* SK (Marcum & Kline, 1983; Wohl *et al.*, 1983). Some plasminogens do not form active complexes with SK; however, all active SK–plasmin(ogen) complexes activate all plasminogen species. Conversely, streptokinases isolated from different streptococcal strains exhibit variation in their response to plasminogens (Ellis & Armstrong, 1971; McCoy *et al.*, 1991).

Schaller *et al.* (Marti *et al.*, 1985; Schaller *et al.*, 1992) have correlated the *S. equisimilis* SK-mediated activation of different mammalian plasminogens with their primary sequences and have proposed three residues of the human plasminogen B-chain as participating in the streptokinase binding site (SK-BS): Arg_{610}, Arg_{644}, and/or Ser_{722}. This approach assumes that the observed species dependency is due to structural differences in the SK-BS and not to structural differences in the plasmin(ogen)–SK activator active site, which would result in a complex possessing no activity. Other investigators place the SK-BS in the vicinity of the plasminogen potential active site (Wohl, 1984) and/or its activation bond (Summaria *et al.*, 1982).

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[1] Abbreviations: Glu-plg or Glu-plasminogen, native plasminogen with NH_2-terminal glutamic acid (Glu_1–Asn_{791}); Lys-plg or Lys-plasminogen, proteolytically modified form of plasminogen, predominantly residues Lys_{78}–Asn_{791} with NH_2-terminal lysine; NTP, NH_2-terminal peptide of plasminogen, predominantly residues Glu_1–Lys_{77}; SK, streptokinase; SK-BS, streptokinase binding site on plasminogen; tc-u-PA, two-chain urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; SE-HPLC, size-exclusion high-performance liquid chromatography; S-2251, D-valyl-L-leucyl-L-lysine-*p*-nitroanilide; 6-AHA, 6-aminohexanoic acid; τ_E, molecular elution time; CHO, Chinese hamster ovary; mAb, monoclonal antibody; DMEM, Dulbecco's modified Eagles medium; ELISA, enzyme-linked immunosorbent assay; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); MPA, mycophenolic acid; IgG, immunoglobulin G.

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In the present paper we report that a plasminogen variant containing a single mutation in the serine protease domain ($Arg_{719} \rightarrow$ Glu) has a significantly lower affinity for streptokinase than the wild-type molecule and therefore has a reduced ability to form active streptokinase–plasminogen complexes.

EXPERIMENTAL PROCEDURES

Cloning of Plasminogen. Plasminogen cDNA was cloned from a human liver cDNA library in λGT11 obtained from Clontech (Palo Alto, CA) using a random hexanucleotide-labeled partial clone. The cDNA identified codes for a Glu-plasminogen protein of 791 amino acids, identical to those reported by Forsgren *et al.* (1987).

Mutagenesis. Site-directed mutagenesis was performed on the 0.85-kb *Eco*RV to *Hin*dIII fragment of plasminogen cloned into M13mp18. Mutagenesis to substitute glutamic acid for arginine at residue 719 was directed using a 24-base oligonucleotide, 5′ GGTGGATTGGACTTCTCCATTCAG 3′, following the procedure of Kunkel *et al.* (1987). After DNA sequencing, the resulting mutant was cloned as an *Eco*RV to *Sph*I fragment replacing the corresponding wild-type sequence in plasminogen. The expression vector pGW1HG places the mutant plasminogen cDNA under the control of the hCMV·MIE promoter and the SV40 polyadenylation signal. Selection of stable cell lines was achieved using the bacterial xanthine–guanine phosphoribosyl transferase gene under the control of an SV40 promoter.

DNA Transfection and Protein Production. *Not*1-linearized plasmid DNA (40 μg) was introduced into Chinese hamster ovary K1 (CHO-K1) cells by electroporation (Neumann *et al.*, 1982) using 800 V and 25 μF. A selective medium (250 μg cm^{-3} xanthine, 5 μg cm^{-3} mycophenolic acid (MPA), and 1× hypoxanthine–thymidine) was added 24 h post transfection, and the media were changed every 2–3 days. Plates yielding MPA-resistant colonies were screened for plasminogen production using an ELISA (see below). The highest producing line was scaled up in 850-cm^3 roller bottles and, when confluent, harvested every 24 h [Dulbecco's modified Eagles medium–F12 (DMEM–F12), 2 mM glutamine, 10 mM sodium bicarbonate, and 20 KIU cm^{-3} aprotinin], yielding 1 μg cm^{-3} plasminogen. Protein was purified using lysine–Sepharose chromatography (Deutsch & Mertz, 1970).

Plasminogen ELISA. ELISA microtiter plates (Linbro EIA-II, Flow Laboratories, Irvine, U.K.) were coated with 100 μL/well of polyclonal anti-human plasminogen antiserum (sheep IgG, PC065, The Binding Site, Birmingham, U.K., or rabbit antiserum, in-house) diluted 1:1000 in bicarbonate coating buffer (0.015 M Na_2CO_3/0.035 M $NaHCO_3$, pH 9.6) and incubated overnight or longer at 4 °C. Coating solution was removed, and the wells were washed with three changes of 0.05 M phosphate/0.1 M NaCl/0.1% (v/v) Tween-80 buffer, pH 7.5 (PBS/Tween). Samples and plasminogen standards (Glu- and Lys-plasminogen concentrations were determined spectrophotometrically; see below) were diluted in PBS/Tween, 100 μL was added per well, and the solutions were incubated at 37 °C for 1 h. Wells were washed three times in PBS/Tween prior to the addition of 100 μL/well of monoclonal anti-plasminogen (American Diagnostica Inc., New York), diluted 1:1000 in PBS/Tween, and incubated at 37 °C for 1 h. Wells were again washed three times with PBS/Tween and 100 μL/well of horseradish peroxidase-conjugated goat anti-mouse IgG (Tago Immunologicals, Burlingame), diluted 1:1000 in PBS/Tween per well, and incubated at 37 °C for 1 h. Excess anti-mouse reagent was removed by three washes with PBS/Tween, bound peroxidase detected with the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate (Sigma, Poole, U.K.), and the absorbance measured at 405 nm using a Multiscan 96-well plate reader (Flow Laboratories, Irvine, U.K.). Plasminogen concentration was determined from a linear log plot of absorbance versus concentration.

The concentration of recombinant plasminogen in expression medium was determined using a mixture of four monoclonal anti-plasminogen antibodies against different epitopes within the molecule, while epitope recognition within purified recombinant plasminogen was carried out with each separately. The American Diagnostica Inc. monoclonal antibodies used were no. 3641 against the amino-terminal peptide (NTP), no. 3642 against kringles 1–3 (K1–3), no. 3647 against kringle 4 (K4), and no. 3644 against the kringle 5–protease domain (K5–SP).

Proteins. Nonrecombinant human plasminogen samples were purchased from the Plasma Fractionation Laboratory, Churchill Hospital (Oxford, U.K.) and Biopool (Umeå, Sweden) and obtained from Kabi Pharmaceuticals (Stockholm, Sweden). Plasminogen was also isolated from plasma Cohn fraction III (Plasma Fractionation Plant, Elstree, U.K.) on lysine–Sepharose essentially as described by Deutsch and Mertz (1970). Protein concentrations were determined spectrophotometrically at 280 nm using $A^{1\%}_{280nm}$ values of 16.8 and 17.4 for Glu-plasminogen (Glu-plg) and Lys-plasminogen (Lys-plg), respectively (Wallén & Wilman, 1972; Christensen, 1988). Albumin-free streptokinase (SK) was a kind gift of Beecham Pharmaceuticals, Epsom, U.K.

Amidolytic Activity Assay. The amidolytic activities of plasmin, formed by two-chain (tc) u-PA activation, and plasmin(ogen)–SK complexes were measured using the chromogenic substrate D-valyl-L-leucyl-L-lysine-*p*-nitroanilide (S-2251). Each plasminogen sample (3 μg cm^{-3} final) was mixed with S-2251 (0.64 mM final) in a reaction buffer of 50 mM Tris–base/100 mM Na_2EDTA/0.005% (w/v) Triton X-100/0.1% (w/v) human serum albumin, pH 7.5 at 37 °C. The reaction was initiated by the addition of either a 1:6 molar ratio of tc-u-PA (30 IU cm^{-3} final) or an equimolar amount of SK (2 μg cm^{-3}), depending on the assay type. At intervals, 100-μL aliquots of the reaction mixture were transferred to wells of a 96-well plate, each containing 25 μL of 4% (v/v) acetic acid which terminated the reaction. Amidolytic activity was measured as an absorbance at 405 nm using a Multiscan 96-well plate reader.

Size-Exclusion High-Performance Liquid Chromatography (SE-HPLC). SE-HPLC experiments were performed using a Hewlett-Packard 1090 HPLC system with diode array detector and fitted with a Tosa Haas TSK G3000SWxl (0.78 × 30 cm i.d.) column, as described in Marshall *et al.* (1994). Samples (25 μL containing approximately 5 μg of plasminogen) were applied to the column equilibrated at 1 cm^3 min^{-1} in 0.1 M sodium phosphate buffer, pH 6.8, with 10 mM 6-aminohexanoic acid (6-AHA) at ambient temperature. Plasminogen–SK complexes were formed in the presence of a 5-fold molar excess of the inhibitor *p*-nitrophenyl *p*-guanidinobenzoate (Chase & Shaw, 1969) in order to prevent autodegradation. The absorbance of the column eluant was monitored at 210 nm as a function of time. The presence of the lysine analogue, 6-AHA, was necessary for the elution of the SK–plasminogen activator complex. This is likely to be a result of disruption by 6-AHA of lysine-dependent interactions within SK–plasminogen oligomers (Ling *et al.*, 1967). Molecular elution times (τ_E) and peak areas were determined

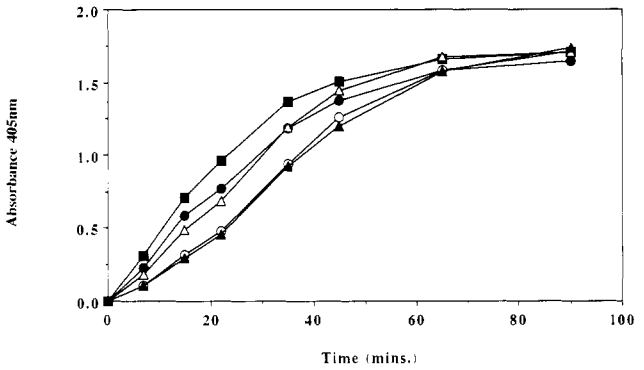

FIGURE 1: Activation by two-chain u-PA of Plg(R719E) (●), plasma-derived wild-type Glu-plasminogen (○, ▲), and two preparations containing mixtures of Glu- and Lys-plasminogen forms (■, △) measured by amidolytic assay.

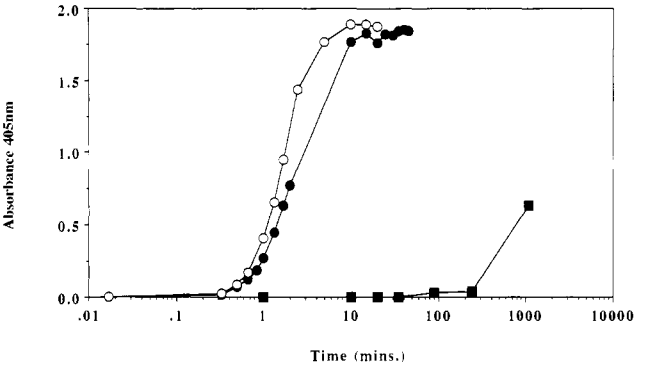

FIGURE 2: SK–plasminogen activator complex formation of wild-type Glu-plasminogen (●), wild-type Lys-plasminogen (○), and mutated Plg(R719E) (■) measured by amidolytic assay.

using a Hewlett-Packard integrator (Marshall *et al.*, 1994). Molecular size estimation using globular protein standards was not pursued since both plasminogen (with 6-AHA) and SK adopt elongated conformations (Marshall *et al.*, 1994, and unpublished observation).

Molecular Modeling. A refined structure of the plasmin serine protease domain was modeled by homology to thrombin using the PPACK/thrombin crystallographic structure (Bode *et al.*, 1989). The homology modeling was performed on a Silicon Graphics Indigo workstation using the Quanta molecular modeling program (Molecular Simulations Inc., Waltham, MA). Sequence alignments were produced using Quanta and the GCG sequence analysis software from the University of Wisconsin (Devereux *et al.*, 1984).

RESULTS

Expression, Purification, and Characterization. Mutated plasminogen $Arg_{719} \rightarrow$ Glu [Plg(R719E)] was constructed by substituting Arg_{719} for glutamic acid using oligonucleotide-directed mutagenesis of the plasminogen cDNA, as described in Experimental Procedures. Successful mutagenesis was confirmed by sequencing the mutated cDNA around the site of mutagenesis, as is usual in these experiments (Davidson *et al.*, 1990), and the mutant cDNA was expressed in a Chinese hamster ovary cell line. The variant was purified from culture medium on lysine–Sepharose. The amino-terminal residue of Plg(R719E) was confirmed as glutamic acid by sequencing methods. The molecular weight of the variant was estimated as 92 000 using SDS–PAGE under reducing conditions. This value shows that Plg(R719E) possesses a similar level of glycosylation as wild-type plasminogen.

The activation of Plg(R719E) by tc-u-PA was indistinguishable from the activation of four wild-type plasminogen preparations (Figure 1). Both variant and wild-type plasminogen samples were found to undergo conformational changes, as measured by SE-HPLC, in the presence of ≥10 mM 6-aminohexanoic acid (Marshall *et al.*, 1994). Using the plasminogen ELISA, Plg(R719E) and wild-type plasminogens were recognized by monoclonal antibodies (mAbs) directed against the NTP, K1–3, K4, and K5–SP fragments. Wild-type Lys-plg was recognized by all mAbs except the mAb which recognizes the NTP (data not shown).

Streptokinase–Plasminogen Complex Formation. The rate of active site formation within SK–plg complexes, as measured by the appearance of amidolytic activity, was rapid for both wild-type Glu-plg and wild-type Lys-plg as expected. However, in this respect the Plg(R719E) variant differed significantly from wild-type Glu-plg. Appearance of amidolytic activity within the SK–Plg(R719E) complex occurred at a rate >100-fold slower than for wild-type Glu-plg (Figure 2).

In order to examine whether this effect was a result of reduced affinity of SK for Plg(R719E), the rates of complex formation were measured by SE-HPLC. Such gel filtration methods have been used successfully to demonstrate SK–plasminogen complexes since 1968 (Taylor & Botts, 1968). Binding of SK to wild-type Glu-plg[2] resulted in a complex which eluted earlier (7.5 min) than wild-type Glu-plg or Plg(R719E) or SK alone (8.3, 8.2, and 8.7 min, respectively) (Figure 3). This indicates that the complex possesses a larger hydrodynamic radius than either Glu-plg or SK. The rate of complex formation, therefore, was measured as the change in absorbance of this early peak. Figure 4 shows that SK possesses a substantially reduced affinity for Plg(R719E) when compared with wild-type Glu-plg; the affinity is also reduced compared with wild-type Lys-plg (data not shown). This reduction in affinity would explain, either wholly or in part, the reduced rate of active site generation as measured using a chromogenic substrate (Figure 2).

DISCUSSION

Streptokinase differs from human plasminogen activators in that it possesses no intrinsic protease activity but acts by forming an active plasminogen activator complex with plasminogen or with plasmin. The regions of plasminogen and streptokinase involved in the formation of this complex have not been elucidated. The binding site for plasminogen appears to reside within the N-terminal 253 residues of streptokinase (Reed *et al.*, 1993), whereas the SK binding site on plasminogen in situated within the C-terminal serine protease domain (Summaria & Robbins, 1976).

During a mutagenesis project on plasminogen it was noted that a single-residue substitution variant [Plg(R719E)], containing glutamic acid at residue 719 in the protease domain, was less readily activated than the wild-type molecule upon the addition of SK (Figure 2). This effect is unlikely to be a result of the expression system *per se*, as recombinant wild-type plasminogen and recombinant thrombin-activatable plasminogen T1 (Dawson *et al.*, 1994) each possess SK activation activities dissimilar to that of Plg(R719E) and similar to that of natural human plasminogen, using the same assay regime (data not shown). The effect is likely to be due either to significant structural perturbation within the activa-

[2] Other data (not shown) acquired on a different HPLC column, on a separate occasion, demonstrate that CHO-derived recombinant wild-type plasminogen possesses similar affinity for SK as natural wild-type plasminogen.

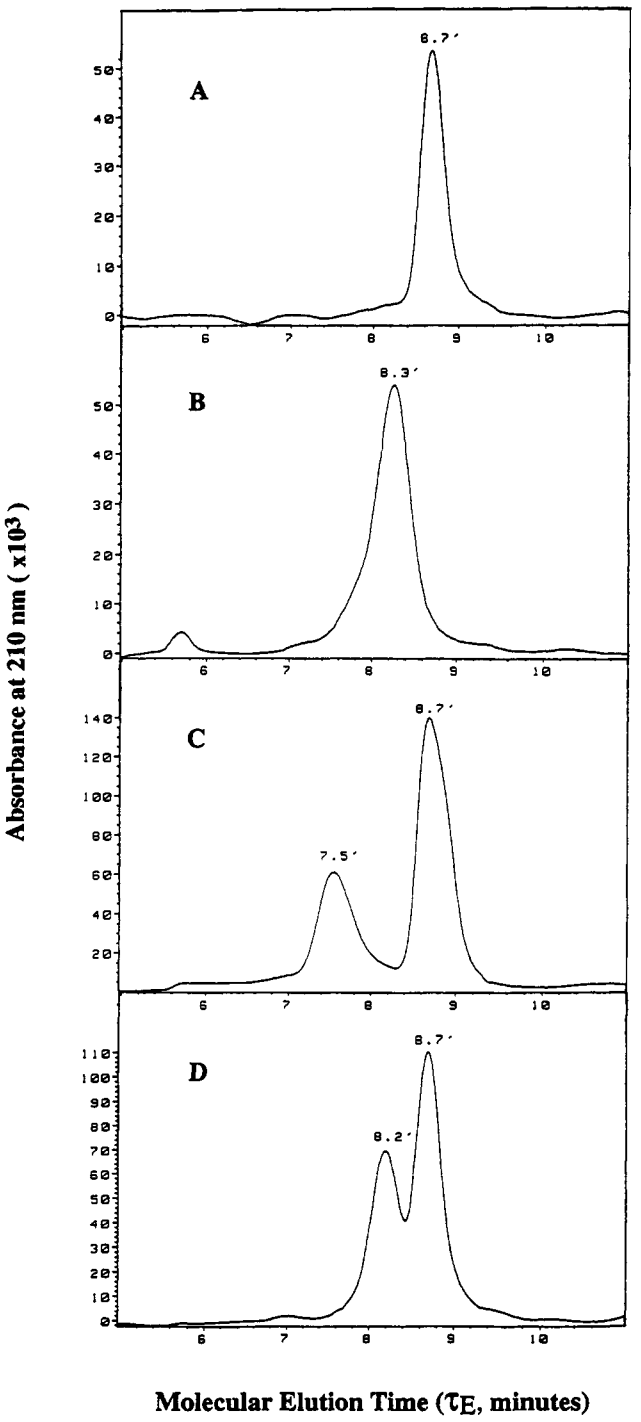

FIGURE 3: Size-exclusion HPLC elution profiles showing (panel A) streptokinase (SK) (τ_E = 8.7 min), (panel B) wild-type Glu-plasminogen (τ_E = 8.3 min), (panel C) the SK/wild-type Glu-plasminogen complex (τ_E = 7.5 min) formed with excess SK (τ_E = 8.7 min), and (panel D) noncomplexed Plg(R719E) (τ_E = 8.2 min) with SK (τ_E = 8.7 min). Data shown for plasminogen/SK mixtures are following 20 min of incubation at room temperature.

tion site or the putative active site of Plg(R719E) or to the substitution of a requisite residue within the SK binding site. The findings were unlikely to be due to gross malfolding of the Plg(R719E) molecule since it was recognized by a range of anti-plasminogen monoclonal antibodies and possessed affinity for lysine–Sepharose. In order to determine whether this mutation resulted in structural perturbation of either the active or activation sites or the SK binding site, both the activation of Plg(R719E) by tc-u-PA and its binding to SK were investigated.

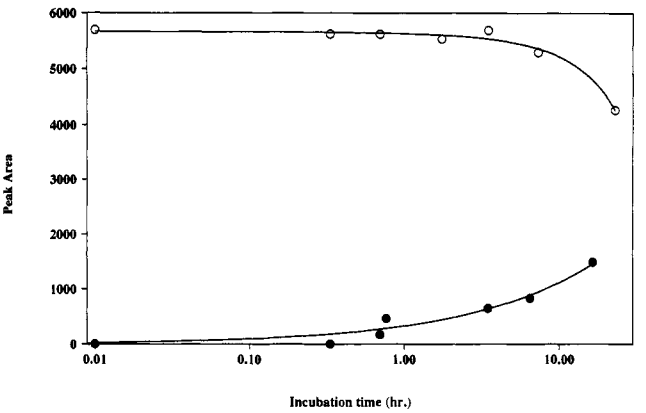

FIGURE 4: Rate of streptokinase complex formation with wild-type Glu-plasminogen (○) and Plg(R719E) (●) measured by size-exclusion HPLC. Reduction of peak areas for wild-type Glu-plasminogen at long incubation times is most likely due to proteolytic degradation of the SK-plasminogen complex. Data shown are representative of three separate experiments.

The activation rate of Plg(R719E) by tc-u-PA was comparable to the activation rates of wild-type plasminogen preparations (Figure 1), demonstrating that the single-residue substitution does not diminish either the ability of the Plg(R719E) activation peptide to be cleaved or the ability of the resultant plasmin (R719E) to form a viable active site. The detrimental effect of mutation at position 719 on the rate of active SK–Plg(R719E) complex formation (Figure 2) must then result from structural alterations within the SK binding site and/or the region involved in the formation of an active site in the wild-type Plg–SK complex.

The binding of Plg(R719E) to SK, as measured by size-exclusion HPLC, was found to be substantially reduced when compared with the wild-type molecule (Figure 4). Although this finding does not directly rule out the possibility that the activity of a viable SK–Plg(R719E) complex differs from that of a complex containing wild-type plasminogen, the reduced affinity of Plg(R719E) for SK implicates human plasminogen Arg_{719} as a binding determinant of the SK binding site (SK-BS).

A model of the plasmin serine protease domain, derived from the known high-resolution structure of thrombin, shows that Arg_{719} is situated at the lip of the putative active site binding pocket in a solvent-accessible position (Figure 5). This may explain previous findings (Wohl, 1984) that the binding of SK to plasminogen, containing benzamidine within this binding pocket, effectively traps the ligand. It is interesting to conjecture that the two glutamic acid residues (714 and 724) in the vicinity of Arg_{719} may be those which, upon chemical modification, result in the abolition of SK affinity (Holloway *et al.*, 1987). Our results do not substantiate earlier claims of the importance of three residues (Arg_{610}, Arg_{644}, and Ser_{722}) in the SK-BS (Marti *et al.*, 1985; Schaller *et al.*, 1992). The positions of these residues in the molecular model are far (>10 Å) both from Arg_{719} and from each other.

Arg_{719} cannot be the only binding determinant of the SK-BS since substitution with glutamic acid was found not to abolish streptokinase binding completely (Figure 4). This is also indicated by conservation of Arg_{719} in bovine plasminogen (Schaller *et al.*, 1985) which does not show activity upon the addition of SK (Marcum & Kline, 1983; Wohl *et al.*, 1983) owing to its inability to bind SK (J. M. Marshall, unpublished result). Additionally, a single streptokinase binding site in the vicinity of Arg_{719} is not able to fully account for the known species dependencies of SK-mediated plasminogen activation

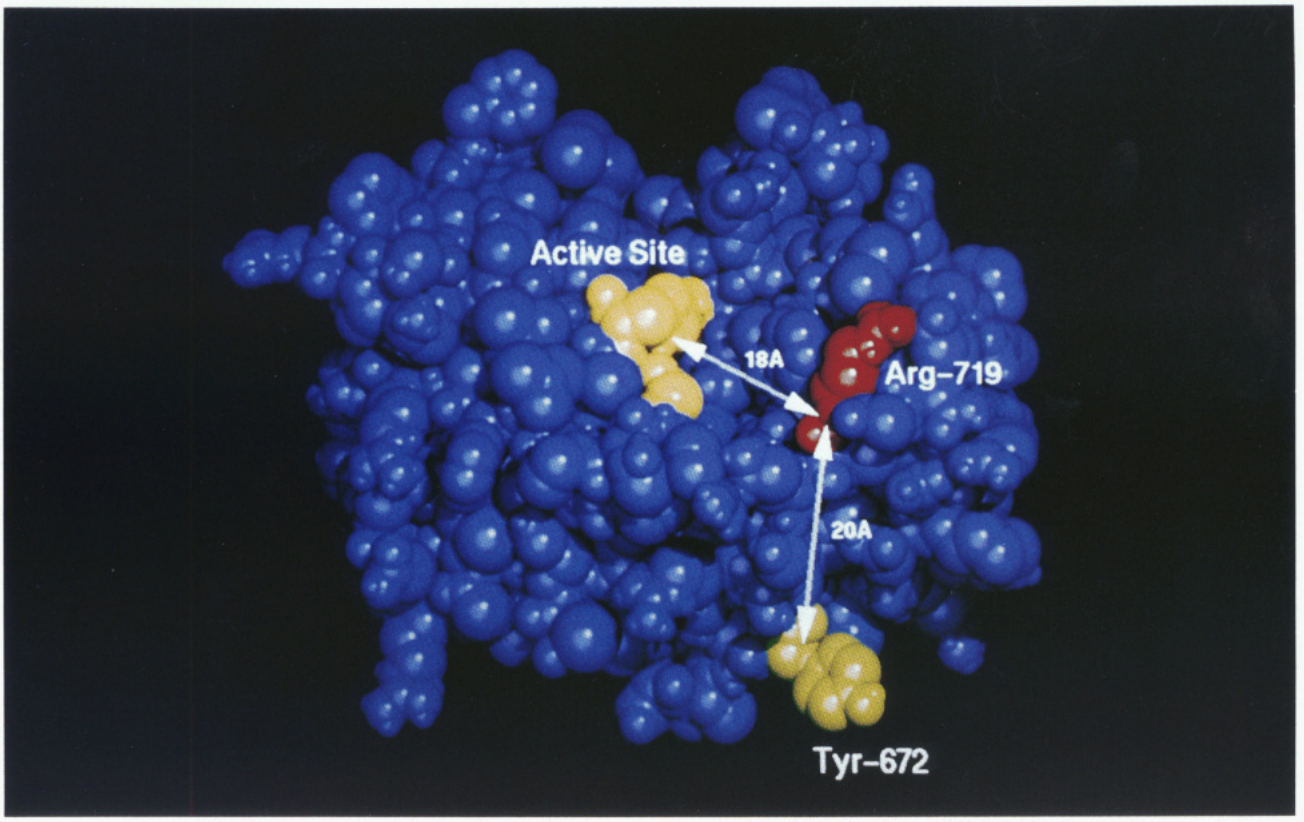

FIGURE 5: A space-filling model of the serine protease domain of plasmin. Highlighted in red is Arg_{719}, in yellow the active site, and in green Tyr_{672}, which is thought to be proximal to the N-terminal domains in the closed conformer of Glu-plasminogen (Bányai & Patthy, 1985).

(Marcum & Kline, 1983; Wohl *et al.*, 1983): there is no obvious correlation between activity with streptokinase and residue types in the neighborhood of Arg_{719} (residues 714–720, 724, and 761). The suggested plasminogen SK-BS also cannot account for the binding of SK to human apolipoprotein [apo(*a*)] (Edelberg *et al.*, 1989), which is a homologue of plasminogen (McLean *et al.*, 1987): apo(*a*) contains a nine-residue deletion in the homologous region to the proposed Arg_{719} plasminogen SK-BS.

In the plasminogen model Arg_{719} is far (>18 Å) from the catalytic triad residues and from the putative oxyanion hole which is generated during activation of serine proteases (Huber & Bode, 1978). It is unlikely, therefore, that binding of SK to the postulated SK-BS directly results in structural changes analogous to the changes observed upon the activation of trypsinogen. A different region of plasminogen, and also therefore of SK, must be critical to the formation of an active center within the complex. This is supported by the results of two recent studies: Reed *et al.* (1993) and Young *et al.* (1993) have independently demonstrated that, to acquire enzymatic activity, plasminogen must interact with at least two independent sites within the streptokinase sequence.

The model (Figure 5) also shows that the putative SK-BS is far from the position of the amino-terminal domains in the compact conformation of Glu-plg (Ponting *et al.*, 1992a,b); Arg_{719} is 20 Å from Tyr_{672} (protease domain) which is able to be cross-linked to Lys_{204} of kringle 2 (Bányai & Patthy, 1985). The Arg_{719} SK-BS, therefore, is likely to be accessible to streptokinase in all plasminogen conformations.

In conclusion, we have established that Arg_{719} in human plasminogen is an important determinant for its interaction with SK. This site, however, cannot fully account for the species specificity of SK, and further studies are required to determine the remaining residues which constitute the SK-BS and which are involved in the generation of enzymatic activity.

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