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Martina L. Sanderson-Smith
University of Wollongong, martina@uow.edu.au

Mark J. Walker
University of Wollongong, mwalker@uow.edu.au

Marie Ranson
University of Wollongong, mranson@uow.edu.au

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The Maintenance of High Affinity Plasminogen Binding by Group A Streptococcal Plasminogen-binding M-like Protein Is Mediated by Arginine and Histidine Residues within the a1 and a2 Repeat Domains.

Abstract

Subversion of the plasminogen activation system is implicated in the virulence of group A streptococci (GAS). GAS displays receptors for the human zymogen plasminogen on the cell surface, one of which is the plasminogen-binding group A streptococcal M-like protein (PAM). The plasminogen binding domain of PAM is highly variable, and this variation has been linked to host selective immune pressure. Site-directed mutagenesis of full-length PAM protein from an invasive GAS isolate was undertaken to assess the contribution of residues in the a1 and a2 repeat domains to plasminogen binding function. Mutagenesis to alanine of key plasminogen binding lysine residues in the a1 and a2 repeats (Lys98 and Lys111) did not abrogate plasminogen binding by PAM nor did additional mutagenesis of Arg101 and His102 and Glu104, which have previously been implicated in plasminogen binding. Plasminogen binding was only abolished with the additional mutagenesis of Arg114 and His115 to alanine. Furthermore, mutagenesis of both arginine (Arg101 and Arg114) and histidine (His102 and His115) residues abolished interaction with plasminogen despite the presence of Lys98 and Lys111 in the binding repeats. This study shows for the first time that residues Arg101, Arg114, His102, and His115 in both the a1 and a2 repeat domains of PAM can mediate high affinity plasminogen binding. These data suggest that highly conserved arginine and histidine residues may compensate for variation elsewhere in the a1 and a2 plasminogen binding repeats, and may explain the maintenance of high affinity plasminogen binding by naturally occurring variants of PAM.

Keywords

Plasminogen, PAM, Streptococcus, Pyogenes, CMMB

Disciplines

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The maintenance of high affinity plasminogen binding by group A streptococcal plasminogen–binding M-like protein (PAM) is mediated by arginine and histidine residues within the a1 and a2 repeat domains*

Martina L Sanderson-Smith, Mark J Walker, Marie Ranson

School of Biological Sciences, University of Wollongong, NSW 2522, Australia.

Address correspondence to: Associate Professor Marie Ranson, School of Biological Sciences, University of Wollongong, Wollongong, NSW, 2522, Australia. Tel: 0061-2-4221 3291; Fax: 0061-2-4221 4135; Email: mranson@uow.edu.au

Subversion of the plasminogen activation system is implicated in the virulence of group A streptococci (GAS). GAS display receptors for the human zymogen plasminogen on the cell surface, one of which is the plasminogen-binding group A streptococcal M-like protein (PAM). The plasminogen binding domain of PAM is highly variable, and this variation has been linked to host selective immune pressure. Site-directed mutagenesis of full length PAM protein from an invasive GAS isolate was undertaken to assess the contribution of residues in the a1 and a2 repeat domains to plasminogen binding function. Mutagenesis to alanine of key plasminogen binding lysine residues in the a1 and a2 repeats (Lys^{98} and Lys^{111}) did not abrogate plasminogen binding by PAM, nor did additional mutagenesis of Arg^{101} and His^{102} and Glu^{104}, which have previously been implicated in plasminogen binding. Plasminogen binding was only abolished with the additional mutagenesis of Arg^{114} and His^{115} to alanine. Furthermore, mutagenesis of both arginine (Arg^{101} and Arg^{114}) and histidine (His^{102} and His^{115}) residues abolished interaction with plasminogen despite the presence of Lys^{98} and Lys^{111} in the binding repeats. This study shows for the first time that residues Arg^{101}, Arg^{114}, His^{102} and His^{115} in both the a1 and a2 repeat domains of PAM can mediate high affinity plasminogen binding. These data suggest that highly conserved arginine and histidine residues may compensate for variation elsewhere in the a1 and a2 plasminogen binding repeats, and may explain the maintenance of high affinity plasminogen binding by naturally occurring variants of PAM.

The Gram positive bacterium *Streptococcus pyogenes* (group A streptococcus, GAS)[1] is responsible for a wide variety of skin and mucosal infections in humans. Current estimates indicate that approximately 1.78 million new cases of severe streptococcal infection occur each year (1). A key feature of invasive GAS infections is the ability of the organism to migrate from cutaneous and mucosal surfaces to deep tissue sites, resulting in severe invasive disease. The binding and activation of plasminogen by GAS has been implicated in the pathogenesis of this organism (2).

Plasminogen is a single chain glycoprotein found in plasma and extracellular fluids at concentrations of approximately 2 μM (3). Cleavage of plasminogen at a single site (Arg^{560}-Val^{561}) by specific plasminogen activators results in the formation of the two-chain plasmin molecule, which contains a serine protease active site in the C-terminal region (4). Human plasminogen can also be activated to plasmin by the GAS protein streptokinase, as part of a highly species specific plasminogen/streptokinase activator complex (2). Plasmin has the ability to degrade fibrin clots, connective tissue and the extracellular matrix (3,4). Thus activation of this proteolytic system by GAS may have significant pathological consequences in the host (5).

Isolated initially from M53 serotype GAS, the plasminogen-binding group A streptococcal M-like protein (PAM) is a 42 kDa molecule which binds both plasmin and plasminogen directly and with high affinity (K_d ~ 1 nM) (6). A newly developed model of GAS infection using mice expressing a human plasminogen transgene indicates that plasminogen plays a critical role in GAS infection, with a significant increase in mortality observed in transgenic mice when compared to wild type littermate control mice (5). Additionally, the PAM-positive GAS isolate AP53 exhibited a 60% increase in mortality in transgenic mice when compared to wild type littermates, and a PAM negative isogenic mutant of this strain showed only minimal virulence in both wild type and human plasminogen transgenic murine backgrounds (5). Thus, it appears that for a subset of GAS isolates, the ability of PAM to focus plasminogen at the GAS cell surface is crucial for virulence.

The major plasmin(ogen)-binding site of PAM is located in the N-terminal variable region of the protein, and is comprised of two characteristic tandem repeats designated a1 and a2. Similar binding motifs have been identified in M-like proteins of other GAS isolates associated with skin infection (6,7). PAM has been implicated in the establishment of GAS skin infections such as impetigo, and the PAM genotype is almost exclusively associated with the chromosomal *emm* pattern D, which is considered a genetic marker for skin tropic GAS (7-9). However, in the Northern Territory of Australia, where streptococcal skin infection is endemic and high rates of invasive infection such as bacteraemia have been reported (10), PAM positive GAS are associated with a variety of disease states (11). The plasminogen binding domain of PAM has been shown to be highly variable, however, this diversity does not abrogate plasminogen binding by naturally occurring PAM variants (12). This finding may, in part, be due to the conservation of key amino acid residues within the plasminogen binding domain (11,12). Plasmin(ogen) is known to interact with its ligands via pre-formed lysine binding-sites within the N-terminally located triple-disulfide bonded kringle domains (4). Kringles 1, 4 and 5 display the highest affinity for lysine-based ligands, with kringle 2 displaying the weakest affinity (13). PAM lacks the typical C-terminal lysine residues characteristic of many plasminogen receptors. Rather, internal lysine residues in the a1 and a2 repeat regions of PAM (Lys^{98} and Lys^{111}) are thought to mediate binding to kringle 2 of plasminogen (14). Early reports suggested that Lys^{98} of the a1 repeat contributed the majority of the plasminogen binding ability of PAM (14). However, studies involving a polypeptide designated VEK-30 and a recombinant kringle 2 modified to contain a high affinity lysine binding site, highlighted a potential role for internal His^{102}, Arg^{101} and Glu^{104} residues within the a1 repeat in this interaction (15,16). VEK-30 is comprised of the six residues preceding the a1 repeat, the entire a1 repeat, together with the first ten residues of the a2 repeat of PAM (17). The relative contribution to binding by residues within the a1 and a2 repeat of the full length PAM protein to plasminogen has not been characterised.

Here, we have undertaken site-directed mutagenesis studies on the PAM molecule and defined residues involved in the interaction with plasminogen. These studies highlight the important contribution of multiple arginine and histidine residues within both the a1 and a2 plasminogen binding domains and may explain the capacity of naturally occurring PAM variants to maintain high affinity plasminogen binding despite considerable variation observed in the plasminogen binding repeats.

MATERIALS AND METHODS

Bacterial strains and culture methods - *Escherichia coli* TOP10 containing pGEX2T-PAM_{NS13} expression plasmids were grown on Luria Bertani (LB) agar plates or cultured in LB broth supplemented with ampicillin (100 μg/ml) as described previously (18). Plasmid DNA was extracted for PCR and DNA sequence analysis using the Wizard ® *Plus* SV DNA purification kit (Promega, USA).

Site-directed mutagenesis - The PAM gene from streptococcal strain NS13 (PAM_{NS13} GenBank™ AY351851), which is identical to the prototype PAM sequence in the a1 and a2 repeat domain, had previously been cloned into the expression vector pGEX2T (12). To assess the role of specific binding-site residues in the interaction of PAM with plasminogen, site-specific mutations were introduced into the PAM_{NS13} binding site. The wild type construct (100 ng) was used as template DNA to create site-directed mutants with the QuickChange™ site-directed mutagenesis kit (Stratagene, USA). PCR reactions consisted of 1x *Pfu* reaction buffer (Stratagene, USA), 0.25mM dNTPs (Boehringer Mannheim, Germany), 2.5 U of *Pfu* Ultra polymerase (Stratagene, USA), and 125 ng of each primer, made up to a volume of 50 μl with dH_2O. Oligonucleotide Primers (Sigma-Aldrich) were designed as per the manufacturers instructions (Stratagene, USA). In general, primers consisted of sequence encoding the

desired mutation, flanked on either side by fifteen nucleotides of wild type sequence. The specific primer sequences are given in Table 1, with the introduced mutations underlined. For site-directed mutants containing more than two mutations not encoded by a single primer, alanine residues were sequentially introduced using previously mutated DNA as a template. Following an initial denaturation step (95°C, 30 sec), PCR cycling parameters consisted of sixteen cycles of 95°C for 30 sec, 55°C for 1 min and 68°C for 10 min. An additional 7 min extension at 55°C was then performed. PCR was conducted with a Cooled Palm 96 thermocycler (Corbett Research). Non-amplified template DNA was removed by incubation of reactions for 1 h at 37°C following the addition of *Dpn* I (10 U/ 50 µl reaction). *E. coli* TOP10 (Invitrogen, USA) were transformed with 150 ng of *Dpn* I digested PCR product using standard procedures (18).

DNA sequence analysis, expression and purification of recombinant M proteins - DNA sequence analysis was used to verify introduced mutations and confirm the absence of random mutations in site-directed mutants. DNA sequence analysis was performed using the primers listed in Table 1, and sequence reactions undertaken using terminator ready reaction mix (PE Applied Biosystems, USA). DNA sequencing gels were prepared as per the manufacturers instructions and electrophoresed using an Applied Biosystems 3130 *X* Genetic Analyser (Applied Biosystems, USA). Sequence data was analysed using ABI Prism™ DNA sequencing analysis software (Perkin Elmer, USA).

Recombinant proteins were expressed and purified essentially as described previously (12,19), from 1 L *E. coli* cultures, using glutathione agarose (Sigma-Aldrich, USA) and native Ni-NTA agarose (Qiagen, Germany) affinity chromatography. Prior to elution from the glutathione agarose column, the GST tag was removed from the N-terminus of recombinant fusion proteins by the addition of one column volume of thrombin solution (1 U thrombin/µl, PBS pH 8.0). The column was incubated for 5 h at room temperature and the cleaved recombinant protein eluted in PBS (pH 8.0). Each step of the protein purification process was monitored by 12% SDS-polyacrylamide gel electrophoresis (PAGE) analysis (20), with protein visualised using Coomassie R 250 staining.

Circular dichroism spectroscopy - To examine potential variation in protein secondary structure as a result of site-directed mutagenesis, far UV Circular dichroism (CD) spectra were obtained for both wild type and mutant recombinant PAM proteins. CD spectra were acquired using a Jasco J-810 Spectropolarimeter (Jasco, Canada) at room temperature. CD spectra data was recorded from 190 – 250 nm in a 1 cm pathlength cell containing 1.5 ml of protein solution at a concentration of 0.04 mg/ml in 10 mM sodium phosphate buffer (pH 7.4). Recorded data represents the average of six scans, corrected for buffer baseline. Molar residue ellipticity ([θ]) was calculated using the following formula:

$[\theta] = \theta \times 100 \times$ Molecular weight / concentration (mg/ml) × distance × Number of amino acids (21) .

The percentage of α-helix was estimated from the ellipticity at 222 nm using the following formula: $\%\ \alpha\text{-helix} = -(\theta_{222\ nm} - 4{,}800)/45{,}400$ (22).

Plasminogen purification and labeling - Glu-plasminogen was purified from human plasma using lysine Sepharose-4B affinity chromatography as described previously (12,23). Purified plasminogen was biotinylated by the addition of 10% (v/v) 1 M $NaHCO_3$ (pH 9), and a 40 molar excess of biotin-X-NHS in dimethyl sulfoxide (Sigma-Aldrich, USA). The reaction was incubated at 4°C overnight with mixing. Free biotin was separated from biotinylated plasminogen by PD-10 gel filtration chromatography (Amersham Biosciences, USA) (12).

Plasminogen binding analysis - Solid phase plasminogen binding assays were performed

in order to assess the impact of introduced mutations on protein function essentially as previously described (12). Ninety-six well microtitre plates (Greiner Bio-one, Germany) were coated with 150 nM recombinant protein (50 µl in 0.1 M $NaHCO_3$) at 4°C overnight. Following three washes with PiNT (50 mM Na_2HPO_4, 150 mM NaCl, 0.05% Tween-80, pH 7.5), plates were blocked with 50 µl of blocking solution (1% skim milk powder, PiNT) for 1 h at 37°C. Wells were washed as above, and 500 nM biotinylated glu-plasminogen was diluted in a three-fold titration across the plate with blocking buffer, in the presence or absence of a 50 fold molar excess of unlabelled glu-plasminogen. Plasminogen was allowed to bind to immobilised proteins for 2 h at room temperature. For competition assays, decreasing concentrations of unlabelled fluid phase wild type PAM_{NS13} (25 µM-0.14 nM) were allowed to compete with immobilised proteins for binding to biotinylated glu-plasminogen. Competitor was titrated three-fold across the microtitre plate prior to the addition of biotinylated glu-plasminogen to all wells, at a final concentration of 500 nM. The assay was incubated for 2 h at room temperature. Following the plasminogen incubation step, microtitre plates were washed three times, and 50 µl of neutravidin conjugated to horse radish peroxidase (Progen, Australia) diluted 1:5000 with blocking solution was added to all wells and incubated for 2 h at room temperature. After five washes with PiNT, the reactions were developed by the addition of 50 µl o-phenylenediamine (Sigma-Aldrich, USA) substrate (8 mM Na_2HPO_4 pH5.0, 2.2mM o-phenylenediamine, 3% H_2O_2). Colour development was stopped by the addition of 50 µl of 10 M hydrochloric acid, and the plates were read at 490 nm using a Spectramax 250 plate reader (Molecular Devices, USA).

Data was normalised against the highest and lowest absorbance value for each assay, and non-linear regression analysis performed using GraphPad® Prism (v4.00, GraphPad software, CA). For the calculation of equilibrium binding dissociation constants (K_d), a one versus two site binding analysis was conducted and the best-fit curve fitted to the data. For competition experiments, a one-site competition curve was fitted to the data from which the effective concentration of competitor required to inhibit binding by 50% (EC_{50}) was calculated.

Statistical analysis - For plasminogen binding experiments, a one-way ANOVA was initially used on all data, followed by an unpaired t-test with Welsch's correction to determine if there was any significant difference in the K_d values for plasminogen binding by PAM mutants and PAM_{NS13}.

RESULTS

The gene encoding wild type PAM_{NS13} shares 100% identity with the prototype PAM sequence in the a1 and a2 repeat domain responsible for the interaction with plasminogen (11). In order to assess the role of specific binding site residues in the interaction of PAM_{NS13} with plasminogen, a number of site-directed mutants were constructed in which the residues of interest were replaced with alanine. The binding site sequences of the five constructed mutants are shown in Fig. 1A. The presence of introduced mutations was verified by DNA sequence analysis. Following expression in *E. coli*, recombinant proteins of approximately 40 kDa in size were purified using glutathione-agarose and Ni-NTA agarose affinity chromatography (Fig. 1B).

The impact of mutations in the a1 and a2 repeat domains of PAM_{NS13} on protein structure were analysed using far UV CD spectroscopy. All proteins used in this study were found to have a CD emission spectrum characteristic of α-helical coiled proteins (24), displaying two characteristic minima at approximately 210 nm and 220 nm, and a maximum peak at 190 nm (Fig. 2). This is similar to the CD spectra of other streptococcal M proteins, which are coiled-coil α-helical proteins (22). Thus, even after mutagenesis these proteins appear to maintain an α-helical secondary structure. Additionally, for all proteins the two minima are of a similar

magnitude, which is indicative of coiled-coil proteins (25). Percent α-helicity ranged from 27% to 44% (Table 2). CD analysis of other streptococcal M proteins has found them to contain between 23% and 70% α-helix (22,26).

PAM_{NS13} has previously been shown to interact with glu-plasminogen with high affinity (K_d ~ 1 nM) (12). To determine the impact of the introduced mutations in the PAM_{NS13} binding site on plasminogen binding, solid phase binding assays were performed utilising biotinylated glu-plasminogen. The recombinant PAM mutants bound plasminogen in a dose-dependant fashion, and saturable binding was achieved with 500 nM plasminogen for 3 of the 5 mutant proteins after 2 h (Fig. 3). Non-linear regression analysis was used to determine the affinity of each recombinant protein for glu-plasminogen (Table 2). Equilibrium dissociation constants (K_d) were calculated using a best-fit non-linear regression curve. The following site-directed mutants where residues were replaced with alanine, $PAM_{NS13}[K^{98}\ K^{111}]$ and $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}]$, bound plasminogen with K_d values of 10.34 nM and 50.24 nM respectively. Whilst this represents a significant decrease in affinity for glu-plasminogen when compared to wild type PAM_{NS13} (Kd = 1.58 nM; $p < 0.05$), binding by these mutants was still specific and saturable. $PAM_{NS13}[R^{101}\ H^{102}]$ bound plasminogen with a K_d value of 1.69 nM, which is not significantly different from that of wild type PAM_{NS13} ($p > 0.05$). Only non-specific plasminogen binding was seen for mutants $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}\ R^{114}\ H^{115}]$ and $PAM_{NS13}[R^{101}\ H^{102}\ R^{114}\ H^{115}]$, indicating that the arginine and histidine residues in both repeat domains are critical residues in the interaction of PAM with plasminogen.

To further explore the contribution of these residues to the interaction with plasminogen, competition binding experiments were performed. The effective concentration of competitor required to inhibit plasminogen binding by 50% (EC_{50}) was determined by fitting a one-site competition curve (Fig. 4). EC_{50} values ranged from 0.25 μM to 22.06 μM. This represents a greater than 90% decrease in EC_{50} values for mutant proteins $PAM_{NS13}[K^{98}\ K^{111}]$, $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}]$ and $PAM_{NS13}[R^{101}\ H^{102}]$, when compared to wild type PAM_{NS13}. As expected, there was an inverse correlation between K_d and EC_{50} (Table 2). These data indicate that whilst mutation of residues Lys^{98}, Arg^{101}, His^{102}, Glu^{104} and Lys^{111} within the plasminogen binding repeats of PAM_{NS13} decreases the avidity of the interaction with plasminogen, the simultaneous mutation of residues Arg^{101}, His^{102}, Arg^{114} and His^{115} is required to fully abolish binding.

DISCUSSION

A key feature of certain strains of *S. pyogenes* is the ability to migrate from cutaneous and mucosal surfaces to deep tissue sites, resulting in serious invasive infections such as bacteraemia, necrotising fasciitis and streptococcal toxic shock-like syndrome. The exact mechanisms of GAS invasive infection have yet to be fully explained, however one hypothesis involves the interaction of GAS with the host plasminogen activation system. Four GAS plasminogen binding proteins have been described in the literature, as well as the secreted plasminogen activator streptokinase (2,27-29). The multiplicity of potential virulence factors associated with *S. pyogenes* that interact with the plasminogen activation system necessitates a deeper understanding of the relationship of GAS with plasminogen.

PAM is a cell surface exposed, high affinity plasminogen receptor expressed by GAS associated with a variety of disease states, and appears to play an integral role in the plasminogen-dependant virulence of PAM positive GAS. In a recent study, it was shown that elimination of PAM dependant plasminogen binding by GAS significantly reduced mortality in mice expressing the human plasminogen transgene (5). Furthermore, whilst the plasminogen binding domain of PAM is highly variable, this variation does not appear to significantly impact on the high affinity interaction of PAM

with plasminogen. The conservation of binding function in spite of sequence diversity is indicative of the physiological significance of this interaction, and the ability of GAS to subvert the host plasminogen activation system (12).

The a1 and a2 repeat domains in the N-terminus of PAM mediate binding to kringle 2 of plasminogen, with the a1 repeat residue Lys^{98} generally considered to make the greatest contribution to this interaction (6,14,17). Using site-directed mutagenesis, this study indicates that residues Arg^{101} and His^{102} within the a1 repeat, together with residues Arg^{114} and His^{115} within the a2 repeat, are critical for the interaction of full length PAM with plasminogen. The interaction with plasminogen of site-directed mutant $PAM_{NS13}[K^{98}\ K^{111}]$ in which residues Lys^{98} and Lys^{111} were mutated to alanines was dose-dependant and specific. Additionally, the K_d value for this interaction was within a physiologically relevant concentration of plasminogen, which circulates in the bloodstream at a concentration of approximately 2 μM (3). Furthermore, mutation of binding site residues Arg^{101}, His^{102}, Arg^{114} and His^{115} abolished plasminogen binding by PAM_{NS13} despite the presence of residues Lys^{98} and Lys^{111}. To our knowledge, this is the first demonstration of a non-lysine-dependent, high affinity interaction between plasminogen and a full-length naturally occurring receptor.

Previous studies involving the interaction of a polypeptide sequence designated VEK-30 with modified kringle 2 of plasminogen highlighted the importance of PAM a1/a2 residues Lys^{98}, Arg^{101}, His^{102}, Glu^{104} and Lys^{111} in the peptide/plasminogen interaction (15,16). Notably, VEK-30 lacks the final three residues of the a2 repeat (Arg^{114}, His^{115}, Glu^{116}). Mutation of residues Lys^{98}, Arg^{101}, His^{102}, Glu^{104} and Lys^{111} in the full-length protein PAM_{NS13} in this study did not eliminate plasminogen binding. However, the avidity of this interaction was significantly reduced, as evidenced by a greater than 90% decrease in EC_{50} values recorded for mutant proteins when compared to wild type PAM_{NS13}. This supports the previous finding that these residues are important in the interaction of PAM with plasminogen. However, the finding that mutation of arginine (Arg^{101} and Arg^{114}) and histidine (His^{102} and His^{115}) residues in both the a1 and a2 repeat was required to fully abrogate plasminogen binding suggests that both repeats are able to mediate high affinity interactions with plasminogen. Residues Arg^{101} and His^{102} of the a1 repeat of PAM have been shown to make numerous saltbridge and hydrophobic electrostatic interactions with recombinant kringle 2, forming a pseudo-ligand similar to the lysine analogue ε- amino caproic acid (16). It is thus likely that the corresponding residues Arg^{114} and His^{115} in the a2 repeat interact with plasminogen in a similar fashion.

The decrease in plasminogen binding by site-directed mutants reported here does not appear to be due to loss of secondary structure, as all site-directed mutants displayed CD spectra characteristic of coiled-coil alpha-helical proteins, similar to that of the wild type PAM_{NS13}. The percent α-helicity for mutants reported here was between 27% and 44%. CD analysis of other streptococcal M proteins has found them to contain between 23% and 70% α-helix (22, 26). However, the spectra for all α-helical proteins are not identical due to the small effect of non-aromatic side chains on the rotary strength of the peptide bond, and occasional helix distortions (24). It has also been shown that the α-helical structure of the peptide VEK-30, representative of the a1 repeat of PAM, increases from 25% to 75% upon binding to kringle 2 of plasminogen (16). It is possible that a similar structural shift could occur in the full length PAM protein. No correlation was seen between percent α-helicity and plasminogen binding function, suggesting that any secondary structure changes have had only a minor influence on the differences in plasminogen binding described here.

We have recently shown that whilst the plasminogen binding domain of PAM is

highly variable, this diversity does not abrogate plasminogen binding by naturally occurring PAM variants (12). In these naturally occurring PAM variants, the Arg and His residues in both a1 and a2 repeat domains are highly conserved (11). Interestingly, in this study, a loss of plasminogen binding was only observed following simultaneous mutation of both the Arg^{101} and His^{102} residues in the a1 repeat, and the Arg^{114} and His^{115} residues in the a2 repeat. Therefore, conservation of Arg and His residues in either repeat domain may compensate for variation elsewhere in the binding repeats, and explain the maintenance of high affinity plasminogen binding by naturally occurring variants of PAM.

Recent findings that the acquisition of plasminogen by *S. pyogenes* may be crucial for the virulence of certain strains of GAS, and the ability of multiple GAS proteins to facilitate this process, necessitates a deeper understanding of the mechanisms via which certain GAS proteins interact with plasminogen. This study highlights for the first time the importance of highly conserved arginine (Arg^{101} and Arg^{114}) and histidine (His^{102} and His^{115}) residues within the binding site of the plasminogen binding protein PAM, in mediating the interaction of this molecule with plasminogen. Such a finding may have implications for the identification of novel plasminogen binding proteins in the future.

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[1] The abbreviations used are GAS, group A streptococcus; PAM, plasminogen binding group A streptococcal M-like protein; PCR, polymerase chain reaction; CD, circular dichroism; LB, Luria Bertani; GST, glutathione S transferase; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate.

Table 1. Forward primers used for PCR construction and DNA sequence analysis of PAM_{NS13} site directed mutants. Underlined sequences indicate introduced mutations. For site-directed mutants containing two or more mutations not encoded in a single primer, mutations were sequentially introduced using previously mutated DNA as a template.

Introduced Mutation	Forward Mutagenesis Primer
K^{98}/A	5'-GATGCTGAGTTGCAACGACTTGCAAACGAGAGACATGAAGAAGCA-3'
K^{111}/A	5'-GAAGCAGAGTTGGAGCGACTTGCAAGCGAGAGACATGATCATGAC-3'
$R^{101}H^{102}E^{104}$/A	5'-CGACTTGCAAACGAGGCAGCAGAAGCAGCAGAGTTGGAGCGA-3'
$R^{101}H^{102}$/A	5'-CGACTTGCAAACGAGGCAGCAGAAGCAGCAGAGTTGGAGCGA-3'
$R^{114}H^{115}$/A	5'-CGACTTGCAAGCGAGGCAGCAGATCATGACAAAAAAGAAGC-3'
Primer	DNA Sequence Analysis Primers
PAMF1	5'-ATAAGCAAGAACATCTTGACGG-3'
PAMR1	5'-CTGTTAATTTCTTGCTTTC-3'
PAMF2	5'-AAAGGGCTTAAGACTGATTTAC-3'
PAMR2	5'-GACCAGCTAATTTGCTGTTTGC-3'
PAMF3	5'-GCAAACAGCAAATTAGCTGCTC-3'
PAMR3	5'-CTTCTCAACATCATCTTTAAGG-3'
pGEX2TF	5'-GGGCTGGCAAGCCACGTTTGGTG-3'
pGEX2TR	5'-CCGGGAGCTGCATGTGTCAGAGG-3'

Table 2. Functional and structural characteristics of PAM_{NS13} site –directed mutants.

Protein	K_d (nM)	EC_{50} (μM)	% α - helix
PAM_{NS13}	1.58	22.06	27
$PAM_{NS13}[K^{98}K^{11}]$	10.34	0.57	41
$PAM_{NS13}[K^{98}R^{101}H^{102}E^{104}K^{111}]$	50.24	0.25	33
$PAM_{NS13}[K^{98}R^{101}H^{102}E^{104}K^{111}R^{114}H^{115}]$	Non-specific binding only	Not determined	44
$PAM_{NS13}[R^{101}H^{102}]$	1.69	4.87	33
$PAM_{NS13}[R^{101}H^{102}R^{114}H^{115}]$	Non-specific binding only	Not determined	29

Fig 1. *A* Alignment of the plasminogen binding domain of wild type PAM_{NS13} with the 5 site-directed mutants constructed in this study. Mutated residues are indicated in bold. *B* 12% SDS-PAGE gel showing the 6 purified recombinant proteins used in this study. Lane 1, PAM_{NS13} ; lane 2, $PAM_{NS13}[K^{98}\ K^{111}]$; lane 3 $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}]$; lane 4, $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}\ R^{114}\ H^{115}]$; lane 5, $PAM_{NS13}[R^{101}\ H^{102}]$; lane 6, $PAM_{NS13}[R^{101}\ H^{102}\ R^{114}\ H^{115}]$. Molecular weight markers are given in kilo Daltons (kDa).

Fig 2. Circular dichroism spectra of recombinant PAM_{NS13} mutants. *A* PAM_{NS13}; *B* $PAM_{NS13}[K^{98}\ K^{111}]$; *C* $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}]$; *D* $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}\ R^{114}\ H^{115}]$; *E* $PAM_{NS13}[R^{101}\ H^{102}]$; *F* $PAM_{NS13}[R^{101}\ H^{102}\ R^{114}\ H^{115}]$ All proteins exhibit CD emission spectrum characteristic of α-helical coiled proteins (24), displaying two characteristic minima at approximately 210 nm and 220 nm, and a maximum peak at 190 nm

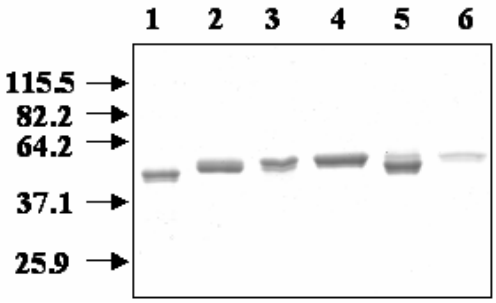
Fig 3. Saturation binding analysis of biotinylated glu-plasminogen to immobilised recombinant PAM_{NS13} mutant proteins. Biotinylated glu-plasminogen binding to immobilised recombinant protein (*A* PAM_{NS13}; *B* $PAM_{NS13}[K^{98}\ K^{111}]$; *C* $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}]$; *D* $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}\ R^{114}\ H^{115}]$; *E* $PAM_{NS13}[R^{101}\ H^{102}]$; *F* $PAM_{NS13}[R^{101}\ H^{102}\ R^{114}\ H^{115}]$) was measured in the absence (▼, total binding) and presence (●, non-specific binding) of a 50 fold molar excess of unlabelled glu-plasminogen. Specific binding (■) was determined by subtracting non-specific binding from total binding at each concentration. A one-site hyperbolic binding function was fitted to the data ($p < 0.05$), from which the binding dissociation constants were determined. Error bars represent the standard error of the mean (SEM; $n = 3$).

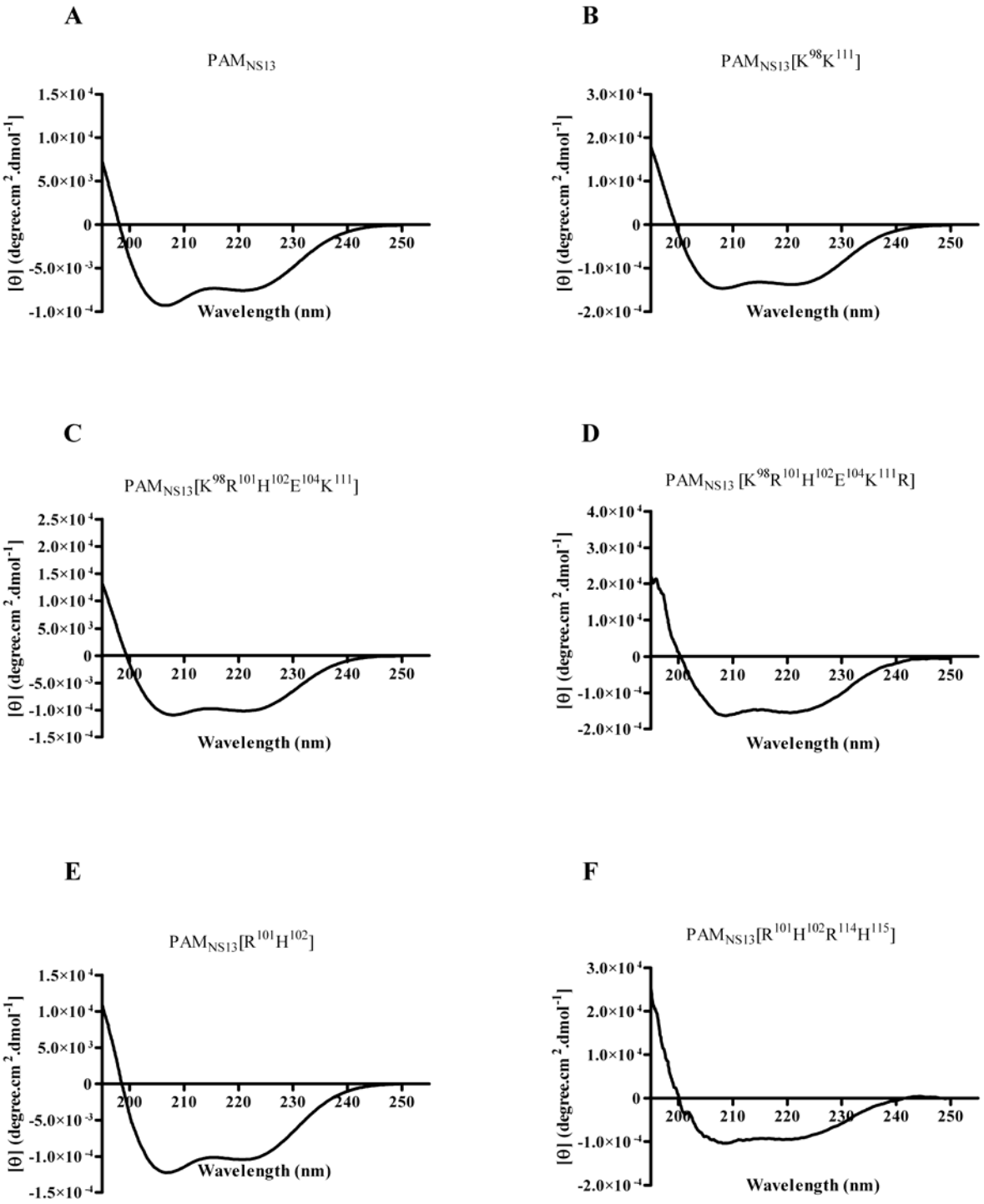
Fig 4. Competition of glu-plasminogen binding to immobilised recombinant PAM variants with fluid phase PAM_{NS13}. Binding of biotinylated glu-plasminogen to immobilised *A* PAM_{NS13}, *B* $PAM_{NS13}[K^{98}\ K^{111}]$; *C* $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}]$, and *D* $PAM_{NS13}[R^{101}\ H^{102}]$ was measured in the presence of varying concentrations of unlabelled fluid phase PAM_{NS13}. Data points are the mean values of triplicate readings, with error bars indicating the standard error of the mean (SEM; n=3). One-site competition analysis was performed on data for all recombinant proteins; this analysis was used to determine the concentration of PAM_{NS13} required to inhibit binding of biotinylated glu-plasminogen by 50% (EC_{50}).

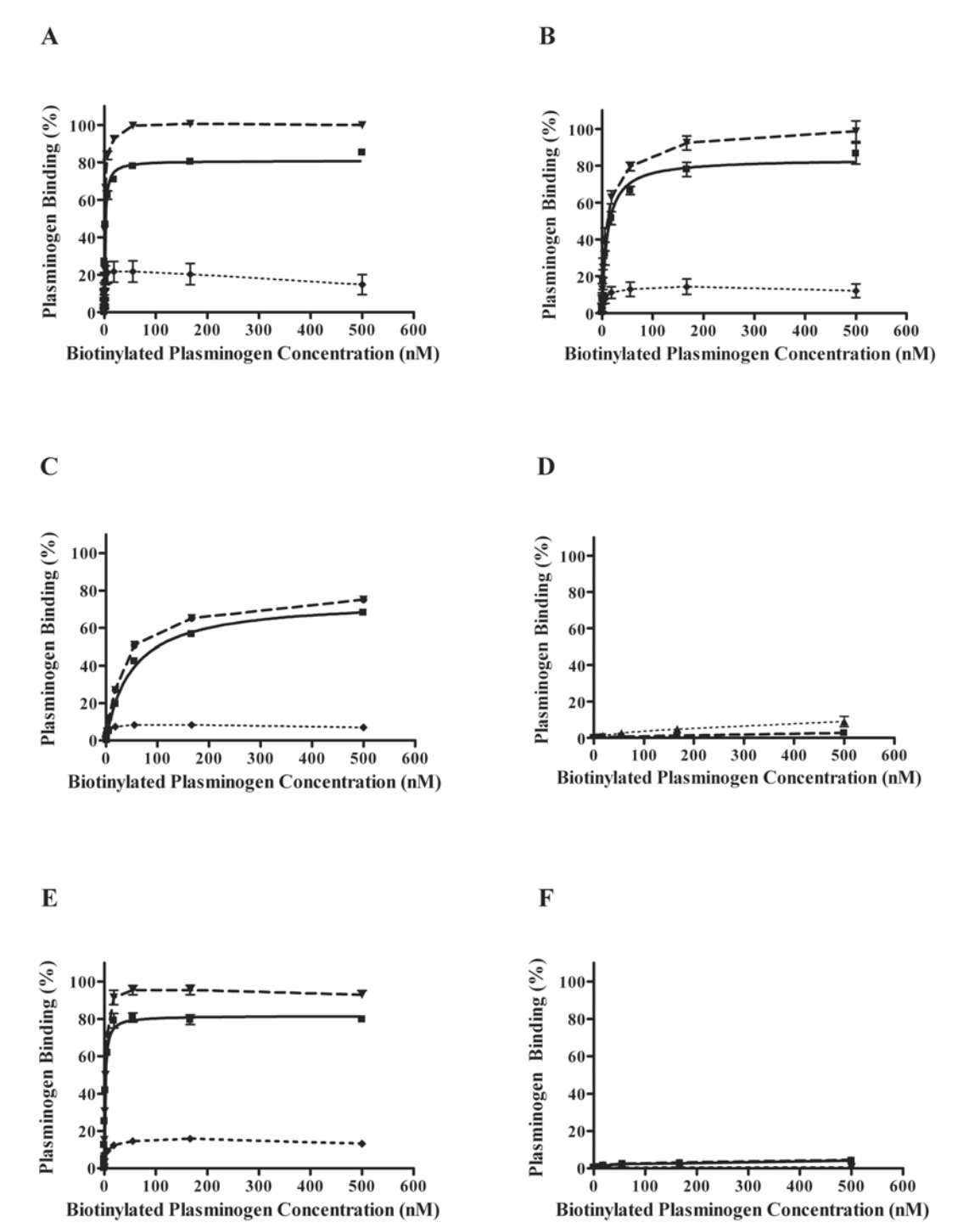
A

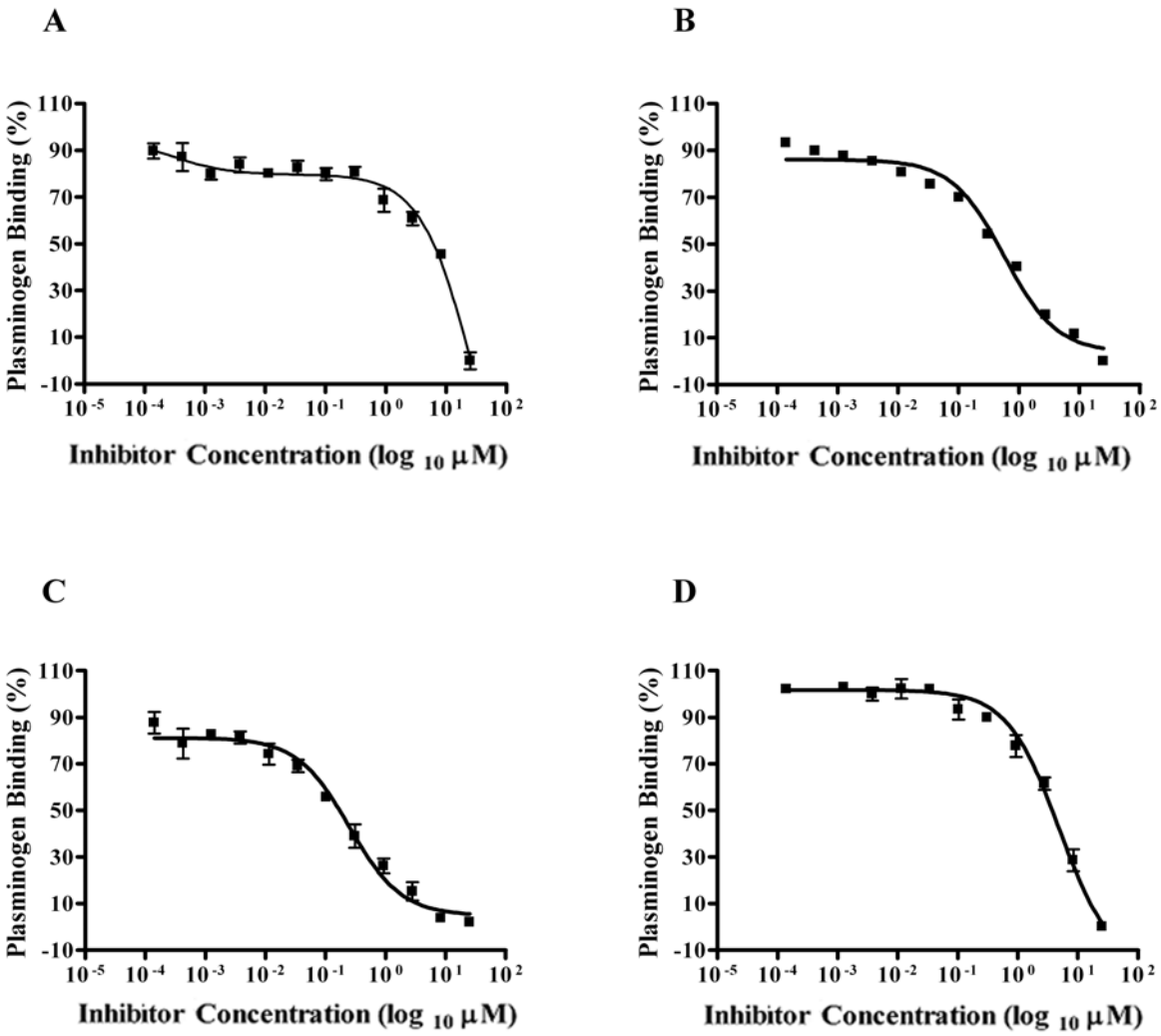
Recombinant Protein	Plasminogen Binding Site	
	a1	a2
PAM_{NS13}	DAELQRLKNERHE	EAELERLKSERHD
$PAM_{NS13}[K^{98}\ K^{111}]$	DAELQRL**A**NERHE	EAELERL**A**SERHD
$PAM_{NS13}[K^{98}R^{101}H^{102}E^{104}K^{111}]$	DAELQRL**A**NE**AA**E	**A**AELERL**A**SERHD
$PAM_{NS13}[K^{98}R^{101}H^{102}E^{104}K^{111}R^{114}H^{115}]$	DAELQRL**A**NE**AA**E	**A**AELERL**A**SE**AA**D
$PAM_{NS13}[R^{101}H^{102}]$	DAELQRLKNE**AA**E	EAELERLKSERHD
$PAM_{NS13}[R^{101}H^{102}R^{114}H^{115}]$	DAELQRLKNE**AA**E	EAELERLKSE**AA**D

B




A
PAM$_{NS13}$
B
PAM$_{NS13}$[K^{98}K^{111}]
C
PAM$_{NS13}$[K^{98}R^{101}H^{102}E^{104}K^{111}]
D
PAM$_{NS13}$ [K^{98}R^{101}H^{102}E^{104}K^{111}R]
E
PAM$_{NS13}$[R^{101}H^{102}]
F
PAM$_{NS13}$[R^{101}H^{102}R^{114}H^{115}]
[θ] (degree.cm^2.dmol^{-1}]
Wavelength (nm)


A
B
C
D
E
F
Plasminogen Binding (%)
Biotinylated Plasminogen Concentration (nM)
0
20
40
60
80
100
0
100
200
300
400
500
600


A
B
C
D
Plasminogen Binding (%)
110
90
70
50
30
10
-10
10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2}
Inhibitor Concentration (log $_{10}$ μM)