Contribution of Conserved Lysine Residues in the α₂-Antiplasmin C Terminus to Plasmin Binding and Inhibition*

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α₂-Antiplasmin is the physiological inhibitor of plasmin and is unique in the serpin family due to N- and C-terminal extensions beyond its core domain. The C-terminal extension comprises 55 amino acids from Asn-410 to Lys-464, and the lysine residues (Lys-418, Lys-427, Lys-434, Lys-441, Lys-448, and Lys-464) within this region are important in mediating the initial interaction with kringle domains of plasmin. To understand the role of lysine residues within the C terminus of α₂-antiplasmin, we systematically and sequentially mutated the C-terminal lysines, studied the effects on the rate of plasmin inhibition, and measured the binding affinity for plasmin via surface plasmon resonance. We determined that the C-terminal lysine (Lys-464) is individually most important in initiating binding to plasmin. Using two independent methods, we also showed that the conserved internal lysine residues play a major role mediating binding of the C terminus of α₂-antiplasmin to kringle domains of plasmin and in accelerating the rate of interaction between α₂-antiplasmin and plasmin. When the C terminus of α₂-antiplasmin was removed, the binding affinity for active site-blocked plasmin remained high, suggesting additional exosite interactions between the serpin core and plasmin.

When tissue injury occurs, fibrinolysis and coagulation are activated in concert. Tissue plasminogen activator secreted by the injured endothelium activates plasminogen to plasmin, which in turn degrades the fibrin clot. Fibrinolysis remains localized because tissue plasminogen activator and plasminogen co-localize on fibrin strands, dramatically improving catalytic efficiency (1). In addition, free plasmin in plasma is rapidly inactivated by its principal regulator, α₂-antiplasmin. Dysregulation of either fibrinolysis or coagulation has the potential to cause thrombosis, whereas on the other hand, therapeutic manipulation of these pathways can be used to treat thrombotic diseases (2). Currently, most therapeutic agents used to promote fibrinolysis directly activate plasminogen, but their use is limited by the risk of excessive bleeding. However, there is increasing evidence that α₂-antiplasmin is a useful alternative target in the development of new therapeutics for thrombotic diseases (3).

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**EXPERIMENTAL PROCEDURES**

**Construction of α2-Antiplasmin Variants—**Human WT α2-antiplasmin cDNA was isolated from a liver cDNA library using PCR with primers 5′-GGA TCC ACC CCA GGA GCA GGT GTG CC-3′ and 5′-GGA TCC TCA CTT GGG GCT GCC AAA C-3′. The product was cloned into the pET-His(3a) expression vector and sequenced for authenticity (12). The QuikChange site-directed mutagenesis kit (Stratagene) was used on human WT α2-antiplasmin template in which alanine or stop codons were introduced at various lysine residues along the C terminus. Several mutations within the C terminus of human α2-antiplasmin were made as follows: K427A, K434A, K441A, K448A, K464A, P414stop (CtermΔ), K448A/K464A, K434A/K448A/K464A, K434A/K441A/K448A/K464A, K434A/K441A/K448A/K464WT, L449stop, and K448A/L449stop (see Fig. 1). All constructs were nucleotide-sequenced to confirm the mutations introduced.

**Expression and Purification of α2-Antiplasmin Variants—**Recombinant human α2-antiplasmin (WT and mutants) was expressed in Escherichia coli BL21(DE3)pLysS cells. Cells were grown overnight at 37 °C with shaking at 220 rpm in 2X tryptone-yeast culture medium supplemented with 50 μg/ml ampicillin. The cell cultures were then diluted 1:10 in fresh 2X tryptone-yeast medium supplemented with 50 μg/ml ampicillin and grown for a further 2 h at 37 °C and 220 rpm. Human α2-antiplasmin was then induced with a final concentration of 0.02 mM isopropyl β-D-thiogalactopyranoside and incubated for 4 h at 30 °C and 220 rpm. Cells were harvested by centrifugation at 3500 rpm (Beckman JS-4.2 rotor) for 20 min and stored at −80 °C until used.

To obtain soluble recombinant α2-antiplasmin, the cell pellet was lysed in lysis buffer (50 mM NaPO4 (pH 8.0), 500 mM NaCl, 20 mM imidazole, and 5 mM β-mercaptoethanol) containing 1 mg/ml lysozyme, 0.2 mg/ml DNase, 1:1000 protease inhibitor mixture, and 0.01% phenylmethanesulfonyl fluoride per liter of cell culture. The cells were frozen in liquid nitrogen and then completely thawed three times in a 37 °C water bath. The lysate was centrifuged (Sorvall SS34 rotor) at 18,000 rpm for 20 min. To purify recombinant α2-antiplasmin, the supernatants were loaded onto a HisTrap column (GE Healthcare) and eluted with a linear imidazole gradient (0.02–0.5 M) in lysis buffer. The proteins were further purified on a Mono Q column (GE Healthcare) with a linear NaCl gradient (0–0.5 M) in 20 mM Tris (pH 8.0) and 0.1 mM EDTA. All recombinant human α2-antiplasmin were electrophoresed on 12.5% SDS-polyacrylamide gel to confirm the purity of peak fractions. Aliquots of recombinant protein were stored at −80 °C until used.

**Determination of Stoichiometry of Inhibition—**The stoichiometry of inhibition (SI) was determined by incubating 1 nM recombinant protein were stored at −80 °C until used. The rate of plasmin inhibition by recombinant α2-antiplasmin was determined using a continuous method described previously (13). Plasmin (0.5 nM) was reacted with various concentrations of recombinant α2-antiplasmin, WT (1.0–2.5 nM) or mutant (1.5–40 nM), in the presence of 1 mM H-Ala-Phe-Lys-AMC at 25 °C. Fluorescence emission was continuously measured using a FLUOstar Optima plate reader at 355/460 nm over time. Tripletrials were performed with each recombinant α2-antiplasmin in 20 mM Tris (pH 8.0), 150 mM NaCl, and 0.01% Tween 20 in a 1% bovine serum albumin-coated PerkinElmer OptiPlate.

**Measuring the Rate of Plasmin Inhibition via Kinetic Assay—**The rate of plasmin inhibition by recombinant α2-antiplasmin was determined using a continuous method described previously (13). Plasmin (0.5 nM) was reacted with various concentrations of recombinant α2-antiplasmin, WT (1.0–2.5 nM) or mutant (1.5–40 nM), in the presence of 1 mM H-Ala-Phe-Lys-AMC at 25 °C. Fluorescence emission was continuously measured using a FLUOstar Optima plate reader at 355/460 nm over time. Tripletrials were performed with each recombinant α2-antiplasmin in 20 mM Tris (pH 8.0), 150 mM NaCl, and 0.01% Tween 20 in a 1% bovine serum albumin-coated PerkinElmer OptiPlate.

The raw data were fitted using nonlinear regression in GraphPad Prism (Equation 1),

\[
P = \frac{V_0}{k_{obs}} \times (1 - e^{-k_{obs} t}) \quad \text{(Eq. 1)}
\]

where \(P\) is the concentration of product at time \(t\), \(V_0\) is the initial velocity, and \(k_{obs}\) is the apparent first-order rate constant (14).

The rate of plasmin inhibition was adjusted with the SI, substrate concentration ([S]), and Michaelis constant (\(K_m\)) of the substrate to give the second-order rate constant (\(k_a\)) (14).

\[
k_a = k\cdot S (1 + [S] / K_m) \times \text{SI} \quad \text{(Eq. 2)}
\]

where the rate of inhibition was adjusted with the SI, substrate concentration ([S]), and Michaelis constant (\(K_m\)) of the substrate to give the second-order rate constant (\(k_a\)) (14).

**Binding of α2-Antiplasmin to Active Site-blocked Plasmin by Surface Plasmon Resonance—**The interactions between active site-blocked plasmin and various recombinant α2-antiplasmin proteins were determined via surface plasmon resonance using the Biacore T100 system (GE Healthcare). Active site-blocked plasmin was produced by incubating human plasmin with a 1000-fold molar excess of D-Val-Phe-Lys chloromethyl ketone (Calbiochem) at 37 °C for 1 h. Active site-blocked plasmin was then dialyzed overnight at 4 °C in running buffer (0.01 M HEPES (pH 7.4), 0.15 M NaCl, 50 μM EDTA, and 0.05% surfactant P20 (GE Healthcare)). To confirm that the active site was completely blocked, the activity of active site-blocked plasmin was checked against active plasmin in the presence of 200 μM H-Ala-Phe-Lys-AMC fluorogenic substrate.

To measure the binding affinity and rate of plasmin association of α2-antiplasmin with active site-blocked plasmin, we immobilized hexahistidine-tagged WT or mutant α2-antiplasmin on the nitrilotriacetic acid (NTA) surface of a NTA chip (GE Healthcare) following the manufacturer’s instructions. All experiments were carried out in running buffer at a flow rate of 10 μl/min and performed in triplicates. Briefly, the NTA sur-
face was activated by injecting 500 μM NiCl₂ for 1 min. 20 nm WT or mutant recombinant α₂-antiplasmin was immobilized onto the surface on one flow cell for 1 min. A reference flow cell containing no α₂-antiplasmin was used to account for nonspecific binding to the NTA surface. Six different concentrations of active site-blocked plasmin (analyte) were injected for 1 min of association time, followed by 10 min of dissociation time. The range of active site-blocked plasmin concentrations (2–120 nM) was adjusted for each α₂-antiplasmin variant. At the end of each concentration cycle, the NTA surface was completely stripped with regeneration buffer (0.01 M HEPES (pH 7.4), 0.15 M NaCl, containing no EDTA, and 0.05% surfactant P20) at a flow rate of 30 μl/min for 2 min. NTA surface activation and α₂-antiplasmin immobilization were performed at each active site-blocked plasmin concentration. The injection needle was cleaned with an extra wash of running buffer after each subsequent step.

Real-time binding curves were monitored on a sensorgram as resonance units over time. For kinetic and binding affinity analysis of recombinant α₂-antiplasmin with active site-blocked plasmin, we used the two-state reaction model provided in the Biacore T100 evaluation software (Version 1.1.1) (Equation 3).

\[ E + I \rightleftharpoons EI \rightleftharpoons EI^* \]  
(Eq. 3)

This model describes a 1:1 binding of analyte to immobilized ligand, followed by a secondary interaction that stabilizes the two molecules. \(\chi^2\) analysis supported this model. The overall equilibrium dissociation constant (\(K_D\)) was calculated using Equation 4 (15).

\[ K_D = \frac{k_{d1}}{k_{a1}} \times \frac{k_{d2}}{k_{a2} + k_{a2}} \]  
(Eq. 4)

RESULTS

Stoichiometry of Inhibition between Recombinant Human α₂-Antiplasmin and Plasmin—To examine the role of lysine residues in the α₂-antiplasmin C terminus, we employed site-directed mutagenesis and produced a series of mutant recombinant proteins (Fig. 1). Mutations within the C-terminal region of α₂-antiplasmin would not be expected to affect the inhibitory mechanism because this property resides in the serpin core domain. However, to verify that the recombinant proteins were correctly folded, the SI for human plasmin was determined for WT and mutant α₂-antiplasmin.

The serpin/protease ratio that resulted in complete loss of protease activity was determined as the SI of the serpin (Fig. 2). The SI of WT α₂-antiplasmin was found to be 1.0 (Fig. 2, A and E) and corresponds with published values (16). Fig. 2E shows the SI values obtained for each recombinant protein produced. All mutants were shown to have an SI of between 0.9 and 1.5 (Fig. 2, B–E), indicating that the efficiency of mutant protein had not been structurally perturbed by the introduction of various amino acid substitutions in the C terminus. To confirm these observations, WT recombinant α₂-antiplasmin and mutants were also assessed by CD spectrometry. Mutant α₂-antiplasmin proteins produced CD spectra similar to WT α₂-antiplasmin, demonstrating that the mutant proteins retained their native fold (data not shown).

Rate of Plasmin Inhibition of Recombinant Human α₂-Antiplasmin Variants—The primary hypothesis being tested in this work is that lysine residues at the extreme C terminus of α₂-antiplasmin as well as internally within the C-terminal extension accelerate inhibition of plasmin. Therefore, kinetic studies using a protease inhibition (progress curve) assay were performed to measure the rate of plasmin inhibition by WT and mutant α₂-antiplasmin. Fig. 3 shows examples of progress and fitted curves obtained from the analysis of WT and P414stop (CtermΔ) α₂-antiplasmin.

As expected, recombinant human WT α₂-antiplasmin was a fast inhibitor of human plasmin, with a plasmin inhibition rate (\(k_o\)) of (3.7 ± 0.3) \(\times\) 10⁷ M⁻¹ s⁻¹, which corresponds to published results (17). Individual Lys-to-Ala mutations at positions 427, 434, 441, 448, and 464 caused decreases in the rate of plasmin inhibition of 1.5-, 1.6-, 1.7-, 2.8-, and 3.6-fold respectively (Fig. 4) compared with WT α₂-antiplasmin. The K464A mutation produced the greatest reduction in the rate of plasmin inhibition; however, this was modest compared with the effect of removing the entire α₂-antiplasmin C terminus (P414stop), which resulted in a 40-fold reduction (\(k_o = (9.2 \pm 0.2) \times 10^5 \text{ M}^{-1} \text{s}^{-1}\)).

Because the reductions in the inhibition rate observed with individual Lys-to-Ala mutants were small compared with the effect of removal of the complete C terminus, we examined the effect of progressive mutations of the Lys residues in this domain. Compared with α₂-antiplasmin K464A, each additional mutation (K448A/K464A, K441A/K448A/K464A, and K434A/K441A/K448A/K464A) resulted in an ~2-fold reduction in the plasmin inhibition rate (Fig. 5). Therefore, the overall reduction in the rate of plasmin inhibition observed with the four-residue substitution (K434A/K441A/K448A/K464A) was 45-fold, which is comparable with the effect of removing the entire α₂-antiplasmin C terminus (P414stop).
It is conceivable that substitution of four lysine residues within the C terminus of α2-antiplasmin could produce a structural perturbation of the domain. We therefore measured the kinetic effect of adding the lysine analog -aminocaproic acid to the inhibitory reaction. When 1 mM -aminocaproic acid was added to the inhibitory reaction, a $k_a$ of $(2.3 \pm 0.2) \times 10^5$ M$^{-1}$ s$^{-1}$ was observed, which was comparable with the mutant with a deletion of the C terminus (P414stop) ($k_a = (9.2 \pm 0.2) \times 10^5$ M$^{-1}$ s$^{-1}$) (Fig. 4). This suggests that most of the reduction in the plasmin inhibition rate caused by the removal of the C terminus can be accounted for by lysine-specific interactions.

The relative importance of the C-terminal Lys-464 compared with the internal lysine residues was further addressed by measuring the rate of inhibition of a mutant in which Lys-464 was preserved while the internal lysines were mutated to alanine (K427A/K434A/K441A/K448A/K464WT) (Fig. 5). This mutant demonstrated a 5.2-fold rate reduction ($k_a = (7.1 \pm 0.1) \times 10^6$ M$^{-1}$ s$^{-1}$) compared with WT α2-antiplasmin.

The importance of the C-terminal lysine was further evaluated by producing a mutant in which a stop codon was introduced after Lys-448 (L449stop). Despite this mutant possessing a C-terminal lysine, it still showed a rate reduction in plasmin inhibition ($k_a = (1.1 \pm 0.1) \times 10^5$ M$^{-1}$ s$^{-1}$) of 3.4-fold compared with WT α2-antiplasmin. Additionally, when the L449stop mutant was modified by substituting Lys-448 with Ala (K448A/L449stop), there was a further decrease in the plasmin inhibition rate ($k_a = (2.8 \pm 0.1) \times 10^6$ M$^{-1}$ s$^{-1}$).

Binding Affinity of Recombinant Human α2-Antiplasmin Variants for Active Site-blocked Plasmin—The association rate constants described above were derived from measurements of inhibition of plasmin amidolytic activity. To corroborate these results and to partition the α2-antiplasmin/plasmin interaction between the serpin core versus the C-terminal extension, we used surface plasmon resonance to directly measure the binding affinity of recombinant human α2-antiplasmin for human plasmin. WT α2-antiplasmin and various mutants were reacted with active site-blocked plasmin, and binding was observed in real time. The association and dissociation constants were calculated to obtain the binding affinity ($K_D$). Examples of sensorgrams for the interaction between active site-blocked plasmin and WT α2-antiplasmin are shown in Fig. 2.
**α₂-Antiplasmin Binding to Plasmin**

**FIGURE 3. Plasmin inhibition by recombinant human α₂-antiplasmin.** A, progress curves of plasmin inhibition using plasmin (0.5 nM) and WT α₂-antiplasmin (1–2.5 nM) in the presence of H-Ala-Phe-Lys-AMC (1 mM). Nonlinear regression analysis using Equation 1 was used to determine the first-order rate constant (k_{obs}). B, the k_{obs} is plotted against the WT α₂-antiplasmin concentration, and linear regression analysis was performed to determine the corrected second-order rate constant (k'). To account for substrate inhibition, Equation 2 was applied to determine the corrected second-order rate constant (k'). C, progress curves of plasmin inhibition using plasmin (0.5 nM) and P414stop (CtermΔ α₂-antiplasmin (80–140 nM) in the presence of H-Ala-Phe-Lys-AMC (1 mM), the k_{obs} was obtained as described above. D, the k_{obs} is plotted against the CtermΔ α₂-antiplasmin concentration to determine the k'. The k of CtermΔ α₂-antiplasmin was calculated using Equation 2.

and recombinant α₂-antiplasmin are shown in Fig. 6. To achieve similar response units for WT α₂-antiplasmin (Fig. 6A), a higher concentration of active site-blocked plasmin was used with α₂-antiplasmin mutants, which accounts for the difference in the shape of the binding curves observed in the sensorgrams (Fig. 6).

The K_{Dj} of WT α₂-antiplasmin for active site-blocked plasmin was determined to be 1.6 nM, indicating a high affinity interaction. Single Lys-to-Ala mutants at positions 448 and 464 showed decreases in K_{Dj} of 1.3- and 3.3-fold, respectively (Fig. 7), compared with WT α₂-antiplasmin. Removing the C terminus (P414stop) resulted in a 31-fold reduction in K_{Dj} (50 nM). Overall measurements of binding affinity were consistent with association rates observed using the progress curve plasmin inhibition assay.

Sequential mutation of the Lys residues within the C terminus resulted in a progressive decrease in the K_{Dj} which corresponds to the observations made previously. K448A/K464A showed a 2.0-fold reduction in the K_{Dj} (10 nM) compared with K464A. K441A/K448A/K464A produced a 2.8-fold decrease compared with K448A/K464A. An additional 1.5-fold reduction in K_{Dj} was observed with K434A/K441A/K448A/K464A (K_{Dj} = 42 nM). The K_{Dj} for K434A/K441A/K448A/K464A was similar to that obtained by removing the C terminus of α₂-antiplasmin (P414stop). The K_{Dj} for K427A/K434A/K441A/K448A/K464WT was 13 nM, corresponding to a 8.4-fold decrease compared with WT α₂-antiplasmin.

The L449stop mutant demonstrated a K_{Dj} of 6.4 nM, which is similar to that obtained for K464A (K_{Dj} = 5.2 nM). This further confirms what was seen previously with the rate of plasmin inhibition. Subsequent mutation of L449stop to K448A/L449stop resulted in a 17-fold reduction in K_{Dj} (27 nM) compared with WT α₂-antiplasmin.

The rate of plasmin association (k_{a1}) obtained using surface plasmon resonance was very similar to the rate of plasmin inhibition (k_{a}) obtained using the kinetic assay described previously. The dissociation rate constant (k_{d1}) and the forward and reverse rate constants (k_{a2} and k_{d2}) remained relatively unchanged for WT α₂-antiplasmin and mutants with active site-blocked plasmin.

**DISCUSSION**

In this work, we have reported the first comprehensive description of the kinetics of α₂-antiplasmin/plasmin interactions employing two different methods. By incorporating a fluorogenic substrate with high affinity for plasmin, we have been able to use the “progress curve” method to accurately measure the association rates for WT α₂-antiplasmin and mutants. In addition, we have independently observed the α₂-antiplasmin/plasmin interaction via surface plasmon resonance. In both methods, we employed full-length α₂-antiplasmin (WT and mutants) and intact plasmin containing the protease and all kringle domains. It is important to recognize that the two methods measure different kinetic rate constants. Using Fig. 8 as a reference schematic, surface plasmon resonance measures the initial rate of interaction (k_{i}) between α₂-antiplasmin and plasmin. The protease inhibition assay (progress curve) measures the overall rate at which the irreversible covalent α₂-antiplasmin-plasmin complex is formed, resulting in complete inhibition; therefore, this takes into account k_{i}, k_{a}, and k_{d} (Fig. 8). We were able to obtain comparable rates of plasmin inhibition (k_{a}) and assoc-
ciation ($k_a$) despite the fact that both methods measure different values. This indicates that the rapid interaction is predominantly due to the formation of the initial reversible encounter $\alpha_2$-antiplasmin-plasmin complex, thus suggesting that the rate-limiting step occurs when the covalent $\alpha_2$-antiplasmin-plasmin complex is formed.

Using protease inhibition and binding affinity data, we were able to demonstrate a progressive decrease in the rate of plasmin association, inhibition, and binding affinity with consecutive Lys-to-Ala mutations within the C terminus of $\alpha_2$-antiplasmin. We showed that all conserved Lys residues (Lys-427, Lys-434, Lys-441, Lys-448, and Lys-464) play a role in the interaction with kringle domains of plasmin, with Lys-464 being the main initiator, followed by Lys-448, which corresponds with previously published data (6, 10). Individual Lys residues appear to have a minor function in the interaction with plasmin. However, as demonstrated by several of our $\alpha_2$-antiplasmin mutants, primarily K434A/K441A/K448A/K464A and P414stop, we were able to show that when five of the lysines were mutated, the rates of plasmin inhibition and binding were reduced to those of the C-terminally truncated $\alpha_2$-antiplasmin protein. This indicates that the Lys residues within the C terminus of $\alpha_2$-antiplasmin are the primary mediators in the binding to the kringle domains and that removal of these residues will result in the loss of C-terminal binding. Furthermore, K427A/K434A/K441A/K448A/K464WT demonstrated that even with the presence of the most C-terminal lysine with all the internal lysines mutated, the plasmin inhibition rate obtained was not comparable with WT $\alpha_2$-antiplasmin. Therefore, each conserved Lys in the $\alpha_2$-antiplasmin C terminus participates in the binding and inhibition of plasmin.

Previous studies by Frank et al. (6) using individual recombinant plasmin kringle domains (K1, K3, K3mut, K4, and K5) showed that the isolated $\alpha_2$-antiplasmin C terminus had the highest affinity for K1, followed by K4, K5, and K2. In further experiments, Gerber et al. (10) examined the affinity of the recombinant plasmin kringle domains (K1, K1-3, K4, and K4-5). They demonstrated that progressive mutations of lysine residues within the $\alpha_2$-antiplasmin C terminus decreased the affinity for K1-3, although the greatest contribution to binding was attributable to Lys-464 and Lys-448. The apparent lack of effect on the affinity of mutations of Lys-418, Lys-427, Lys-434, and Lys-441 may be explained by the fact that only two lysine-binding kringle domains were present in the K1-3 protein. The seeming discrepancy between our results and those of Gerber et al. can therefore be
They measured the association constants of isolated kringle domains (K1, K4, K1-3, and K4-5) with the C-terminal portion of α2-antiplasmin. By contrast, this study describes the rate of plasmin inhibition and binding affinity of full-length α2-antiplasmin with intact plasmin.

FIGURE 6. Sensorgrams of the binding of recombinant human α2-antiplasmin to active site-blocked plasmin measured by surface plasmon resonance. WT or mutant recombinant α2-antiplasmin (20 nM) was immobilized on a NTA chip. The binding of various concentrations of active site-blocked plasmin to α2-antiplasmin was monitored in real time. A, binding of active site-blocked plasmin (2–8 nM) to WT α2-antiplasmin (χ² = 0.21). B, binding of active site-blocked plasmin (20–120 nM) to P414stop (CtermΔ) recombinant α2-antiplasmin (χ² = 0.60). C, binding of active site-blocked plasmin (4–20 nM) to K464A mutant α2-antiplasmin (χ² = 0.31). D, binding of active site-blocked plasmin (20–100 nM) to K434A/K441A/K448A/K464A mutant α2-antiplasmin (χ² = 0.54).
One striking observation made in this study is that the association rate and binding affinity of plasmin with C-terminally truncated α2-antiplasmin (P414stop) are relatively high ($k_{a1} = 5.1 \times 10^7 \text{ M}^{-1} \text{s}^{-1}; K_D = 49 \text{ nM}$). It is important to note that surface plasmon resonance studies were performed with active site-blocked plasmin, suggesting that the rapid association and high affinity observed in the absence of the C-terminal extension were mediated by exosite interactions between α2-antiplasmin and the plasmin protease domain. These important interactions between the α2-antiplasmin core domain, outside the immediate vicinity of P1-P1’, and the active site cleft of plasmin are also likely to contribute specificity to the serpin/protease interaction. Having additional exosite interactions is not uncommon in the serpin inhibition mechanism, as it may aid in the recognition of its target protein (18, 19). Together with the specific binding of the α2-antiplasmin C terminus to plasmin kringle domains, this leads to an exquisite specificity of the interaction minimizing off-target inhibition of non-cognate proteases.

In summary, we have performed detailed kinetic and binding studies of the interaction between α2-antiplasmin and plasmin. Within the α2-antiplasmin C terminus, we have measured the contribution of the conserved lysines to the interaction with plasmin kringle domains. This study demonstrates that the C-terminal lysine (Lys-464) is the single most important amino acid in this domain. The remaining conserved lysine residues within the C terminus of α2-antiplasmin individually contribute less, but together significantly enhance the rate of association of serpin with plasmin. These data support the zipper model of interaction whereby Lys-464 binds initially to plasmin (most likely at K1), followed by progressive binding of the other conserved lysines (Lys-448, Lys-441, and Lys-434) to the remaining lysine-binding kringle domains (K4, K5, and K2) (6). Our data also highlight the importance of exosite interactions between the α2-antiplasmin core serpin and the plasmin
protease domain, which provide an additional mechanism of specificity in the serpin/protease interaction.

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