Heparin Binding to the Urokinase Kringle Domain†

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ABSTRACT: The binding of urokinase to immobilized heparin and dextran sulfate was studied using activity assays of the bound urokinase. The markedly higher binding observed with high M_r urokinase compared to low M_r urokinase indicated a role for the amino-terminal fragment (ATF). This was confirmed by the use of inactive truncated urokinase and monoclonal antibodies specific for the ATF in competition assays of urokinase binding. Antibody competition assays suggested a site in the kringle domain, and a synthetic decapeptide Arg-52–Trp-62 from the kringle sequence (kringle numbering convention) was competitive in assays of urokinase binding to dextran sulfate and heparin. Heparin binding to the urokinase kringle was unambiguously demonstrated via ^{1}H NMR spectroscopy at 500 MHz. Effective equilibrium association constants (K_a*) were determined for the interaction of isolated kringle fragment and low M_r heparin at pH 7.2. The binding was strong in salt-free ^2H_2O (K_a* ~57 mM^{-1}) and remained significant in 0.15 M NaCl (K_a* ~12 mM^{-1}), supporting a potential physiological role for the interaction. This is the first demonstration of a function for the kringle domain of urokinase, and it suggests that while the classical kringle structure has specificity for lysine binding, there may also exist a class of kringles with affinity for polyanion binding.

The urokinase-type plasminogen activator (u-PA)[1] is now established as an important mediator of cell-surface proteolysis, facilitating several physiological processes of regulated tissue remodeling as well as the pathological invasive behavior of malignant cells (Danø et al., 1985; Pöllänen et al., 1991). This serine proteinase is synthesized and secreted as a single-chain proenzyme (pro-u-PA), which requires specific proteolytic activation before it can efficiently catalyze the activation of plasminogen to plasmin (Petersen et al., 1988). The site of proenzyme activation and hence plasmin generation is the specific high-affinity u-PA receptor located on the cell surface (Blasi, 1988; Stephens et al., 1989). Although it has been shown earlier that u-PA (Pöllänen et al., 1988) and its activation (Pöllänen et al., 1990) are localized at the focal adhesion sites of adherent cells, the mechanism which establishes and maintains this concentration of u-PA on receptors at the adhesion sites during cell migration has not been elucidated. Since it is now known that the u-PA receptor is anchored in the plasma membrane by a mobile lipid anchor (Ploug et al., 1991), other interactions of the receptor protein or its u-PA ligand deserve investigation as possible mediators of the localization mechanism.

It has been shown in cell-free systems that heparin stimulates both the activation of pro-u-PA by plasmin (Lijnen & Collen, 1986; Watahiki et al., 1989) and the activation of plasminogen by u-PA (Andrade-Gordon & Strickland, 1986; Pâques et al., 1986). These effects appear to be mediated by heparin binding to u-PA, although a heparin-binding site has not been identified in the u-PA structure. Recently we demonstrated (Stephens et al., 1991) that brief treatment of rhabdomyosarcoma cells with protamine, a small highly basic polypeptide (Dixon et al., 1986), increases more than 2-fold the amount of u-PA which may be acid-eluted from the cell surface. This result suggested that cell-surface u-PA may be bound via ionic interactions to matrix proteoglycans, as well as through its growth factor domain to the specific u-PA receptor (Blasi, 1988).

We therefore sought data on the structural features of u-PA which mediate interactions with polyanions, and we report that the u-PA kringle domain contains the principal heparin-binding site.

MATERIALS AND METHODS

Urokinase Preparations and Urokinase Fragments. ΔF·u-PA was produced from mouse LB6 cells by transfection with a partial digest of the human genomic u-PA was described previously (Pedersen et al., 1991) and consisted of the first 164 amino acids of the human u-PA sequence, with an additional 30 amino acid sequence produced by a frameshift. ΔF·u-PA was immunopurified from LB6 culture medium using an immobilized monoclonal antibody and analyzed by SDS–PAGE (Pedersen et al., 1991). Only two bands were visible: the major band of 24 kDa (ΔF·u-PA) and a minor band of 28 kDa. Human pro-u-PA was a gift from Collaborative Research Inc. (Bedford, MA)/Sandoz A.G. (Nürnberg, Germany). Its purity was established by SDS–PAGE under reducing and nonreducing conditions. When incubated with plasminogen containing traces of plasmin, it became fully

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[1] Abbreviations: ATF, amino-terminal fragment of u-PA; bFGF, basic fibroblast growth factor; ΔF·u-PA, recombinant inactive truncated u-PA; K_a*, the effective kringle association constant; PSB, phosphate-buffered physiological saline; pro-u-PA, proenzyme form of u-PA; pH*, glass electrode pH reading, uncorrected for deuterium isotope effect; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.

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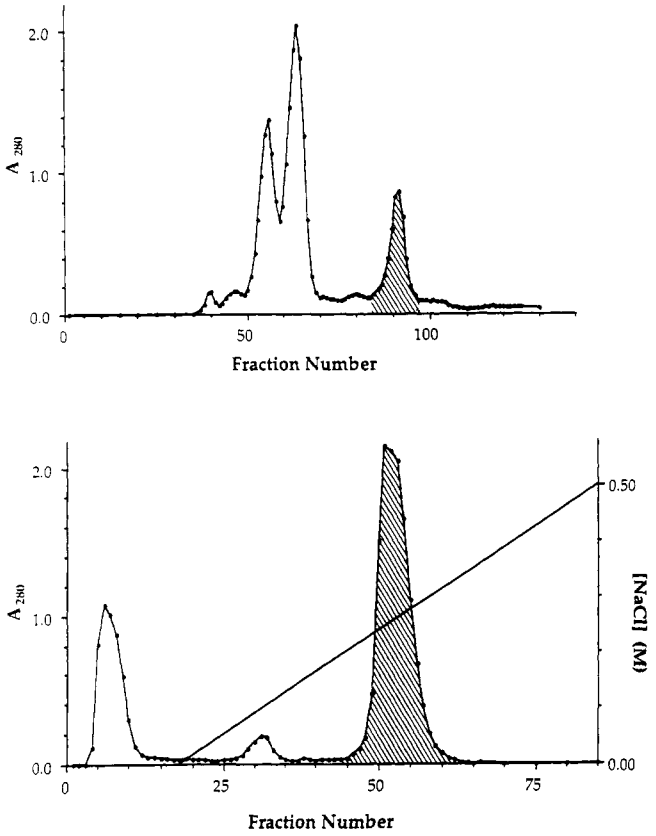

FIGURE 1: Chromatographic purification of the u-PA kringle. Kringle-containing fractions are shown hatched. (A) Plasmin digest fractionation through Sephadex G-100. (B) CM-Sephadex purification of the third major component in (A). The straight line represents the NaCl gradient by reference to the right-hand ordinate.

active in catalyzing the production of plasmin. The u-PA kringle was generated via plasmin digestion of u-PA by a slight modification of the method of Bogusky et al. (1989) as follows: pro-u-PA was dissolved to 17.4 mg/mL in a buffer containing 0.03% (v/v) Tween 80, 11% (v/v) glycerol, 0.2 M NaCl, 0.03 M sodium phosphate, and 0.07 M ammonium bicarbonate, pH 8.2. Plasmin, which cleaves on the C side of Lys-135 in pro-u-PA (Stump et al., 1986a), was added to ~1:200 enzyme/substrate molar ratio. The digestion was allowed to proceed for 48 h at 37 °C and then quenched by cooling to 4 °C. The kringle fragment was purified on Sephadex G-100 (Pharmacia, Uppsala, Sweden) with 0.1 M ammonium bicarbonate and 0.02% NaN_3, pH 8.0, followed by ion-exchange chromatography on CM-Sephadex (Pharmacia) with 0.005 M sodium phosphate followed by a salt gradient, pH 7.5 (Figure 1). The kringle-containing fractions were identified via ^1H NMR by monitoring specific kringle signals such as the characteristic Leu-46 $CH_3^{\delta,\delta'}$ doublets, which appeared at ~−1.0 and ~0.5 ppm, with a splitting of ~6.5 Hz (Thewes et al., 1987; Bogusky et al., 1989). The NMR spectrum and SDS–PAGE criteria established that the kringle sample thus obtained was of high purity (>95%). Several cycles of Edman degradation determined the N-terminal sequence of the kringle fragment to be S-K-T-C-Y-E-G-N-G-H-F-Y-R-G-K-A-S, which corresponds to the Ser-47–Ser-63 stretch of the pro-u-PA sequence (Figure 2). The analysis showed no other free N terminus, indicating sample homogeneity and no further internal cleavage of the domain. By reference to previous studies (Llinás et al., 1983; Thewes et al., 1987), the ^1H NMR spectrum unambiguously indicated that the kringle was properly folded. The yield of u-PA kringle was ~30% theoretical. The plasmin used for

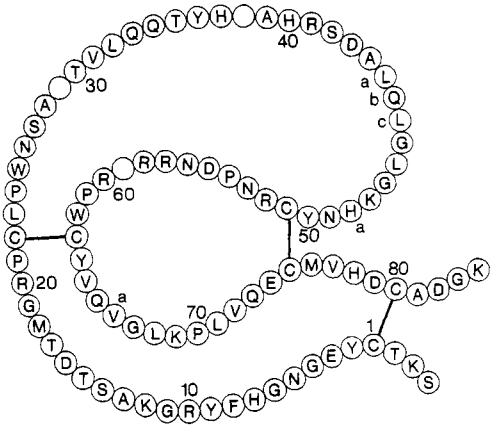

FIGURE 2: Outline of the primary sequence of the u-PA kringle fragment. The sequence corresponds to the Ser-47–Lys-135 segment of pro-u-PA. Three deletions (at sites 29, 38, and 59) and five insertions (at sites 44a–44c, 48a, and 66a) have been introduced to facilitate comparison with other kringles. Site numbering, insertions, and deletions are based on homology with plasminogen kringle 5 (Motta et al., 1987; Thewes, et al., 1987).

the digestion was obtained by activating human plasminogen (Petros et al., 1989) with urokinase (100:1 molar ratio), by incubating the mixture in 0.18 M sodium phosphate, 0.26 M NaCl, 0.13 M glucose, 0.05 M L-lysine, 1.0 mM 6-aminohexanoic acid, and 33% glycerol, pH 7.4, at 4 °C for 4 h. Plasmin activity was assayed with *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride (Chase & Shaw, 1969).

Low M_r heparin (average M_r 3000) was purchased from Sigma (St. Louis, MO). ^2H_2O originated from Merck, Sharp & Dohme, Ltd. (Montreal, Canada). The u-PA decapeptides KP and CP were synthesized and reverse-phase HPLC purified by Dr. Carmela Kantor-Aaltonen at the Department of Biochemistry, University of Helsinki. The purity of each peptide peak was checked by analytical HPLC and confirmed by amino acid analysis. The sequence of the kringle peptide (KP) was R-N-P-D-N-R-R-R-P-W, corresponding to residues 52–62 in the generalized kringle sequence (Figure 2) and residues 104–113 of the u-PA sequence (Figure 4). The sequence of the connecting peptide (CP) was K-P-S-S-P-P-E-E-L-K, corresponding to residues 136–145 in the u-PA sequence (Figure 4). Protamine sulfate (sterile solution for injection, 10 mg/mL) was obtained from Medica, Finland.

u-PA Binding to Immobilized Heparin and Dextran Sulfate. Polystyrene immunoplates (Type 269620, Nunc, Roskilde, Denmark) were treated overnight at 37 °C with 50 μL of a solution of heparin (Medica) or dextran sulfate (M_r 500 000; Pharmacia) in water, so that the wells dried. The coating solution contained 20 IU/mL heparin or 50–500 μg/mL dextran sulfate as shown in the experiments. The wells were then rinsed twice, either with Dulbecco's PBS containing 0.2% bovine serum albumin (BSA) (for dextran sulfate) or with the same buffer diluted 1:3 (for heparin). A third rinse was allowed to stand in the wells for 2 h to block nonspecific binding sites. u-PA (0.5–2 IU/mL) was applied in 50 μL of the rinsing buffer and allowed to bind to triplicate wells for 2 h at 23 °C, after which the wells were rinsed three times. The bound u-PA activity was assayed by incubation with purified human plasminogen and measurement of plasmin thioesterase activity as previously described (Stephens et al., 1987, 1988, 1989). All assays were done with at least triplicate wells, and the results shown are the means obtained in a typical experiment from a series. In competition binding experiments, the u-PA was preincubated with antibodies for 2 h before application

to the polyanion. The following murine monoclonal antibodies to human u-PA were used: 377 and 3921 (American Diagnostica) and clones 6 and 12 (Grøndahl-Hansen et al., 1988). These antibodies have known specificities in the ATF region of u-PA, as detailed below. As a control in some experiments, a murine monoclonal antibody to t-PA was used (ESP-2; American Diagnostica).

^1H NMR Titration Experiments. ^1H NMR spectra were recorded on 1.5 mM u-PA solutions in ^2H_2O, in the Fourier mode at 500 MHz, using a Bruker AM-500 spectrometer. Suppression of the residual $^1H^2HO$ signal was achieved by selectively irradiating the resonance during the 1.5-s relaxation delay period. Typically 160–240 transients were collected for each titration point, using quadrature detection over 32K data points, spectral width 6000 Hz. Chemical shifts of specific resonances were determined by using the peak-picking routine of the Bruker software package and are referred to the sodium (trimethylsilyl)[2,2,3,3-$^2H_4$]propionate resonance using *p*-dioxane as an internal standard (De Marco, 1977).

Determination of Effective Equilibrium Association Constants (K_a).* Ligand (low M_r heparin) titrations were recorded at pH* 7.2, 24 °C, by monitoring the ^1H NMR spectral response to additions of small aliquots of a concentrated (25–50 mM) ligand solution. The titrations were carried out until saturation. Effective equilibrium association constant (K_a*) values were estimated for the heparin/kringle interaction by assuming fast ligand exchange and single kringle-site binding. These assumptions were justified by the observation that the various His resonances monitored to detect binding exhibited parallel trends (indicative of single-site interaction) while yielding a single (averaged) spectrum at each heparin titration point (indicative of fast exchange). These assumptions have been proven adequate when determining the interaction of plasminogen kringles with small ligands (De Marco et al., 1987; Rejante et al., 1991b) and with fibrinogen (Rejante et al., 1991a). Both reciprocal linear plots (De Marco et al., 1987) and nonlinear least-squares fits to the hyperbolic adsorption isotherm profile (Rejante et al., 1991a) were calculated. The two methods agreed within ±3%. In the case of high salt concentration (Figure 12C), it was apparent from inspection of curve C that half-saturation occured at a heparin concentration >3.3 mM. This indicated that at 250 mM [NaCl] the interaction heparin/kringle was relatively weak (K_a* <1 mM^{-1}) and that one could therefore assume that the free ligand concentration was equal to the total ligand concentration (Sahai et al., 1974; Harris, 1986; Rejante et al., 1991b) and justifiably apply the NMR version of the Scatchard plot (Foster & Fyfe, 1969). The heparin titration curves (Figure 12) showed minor deviations from a strict saturable profile, most likely a reflection of heterogeneity in the commercial low M_r heparin and/or residual nonspecific binding. Hence the data was analyzed after subtraction of the nonsaturable linear contributions (Thewes et al., 1990) evident at high [heparin]/[kringle] ratios. The reported K_a* values represent the average of vie independent estimates obtained from an equal number of monitored ligand-sensitive resonances.

RESULTS

Urokinase Binding to Immobilized Dextran Sulfate. High M_r u-PA was able to bind to heparin and dextran sulfate coated microtiter wells, so that u-PA activity was readily measurable in the wells after binding and rinsing. Dextran sulfate was used for most of the experiment of this type, since, as in previous studies, its higher charge density gave stronger

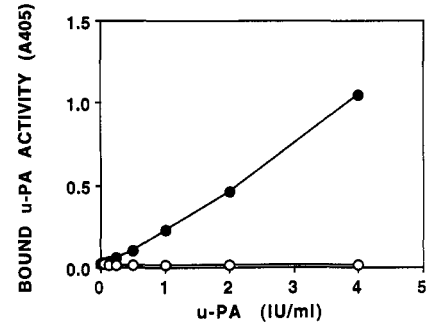

FIGURE 3: Binding of high and low M_r u-PAs to immobilized dextran sulfate. Aliquots (50 μL) of a solution of dextran sulfate in water (500 μg/mL) were allowed to dry in the wells of a microtiter plate during overnight incubation at 37 °C. The wells were then rinsed twice with Dulbecco's PBS containing 0.5% BSA (200 μL), and a third rinse was allowed to stand 2 h in the wells to block nonspecific binding sites. After another rinse, u-PAs (50 μL) diluted in the rinsing buffer to the concentrations shown here added to the wells and allowed to bind for 2 h at 23 °C. After three rinses, purified human plasminogen in u-PA assay buffer [see Stephens et al. (1987)] was added and the plasmin produced was assayed as its thioesterase activity. The activity (A_{405nm}) is shown for bound high (●) and low (○) M_r u-PAs.

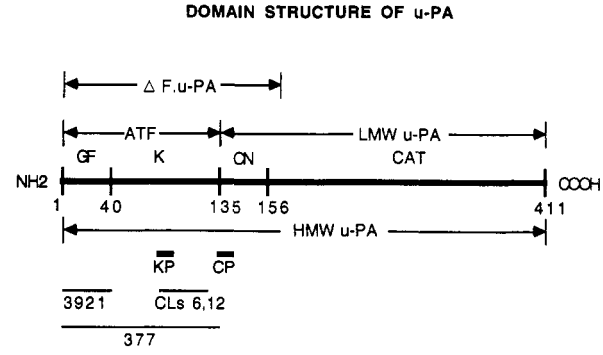

FIGURE 4: Schematic diagram of the domain structure of u-PA. The domain structure of high M_r u-PA (HMW u-PA) is shown, illustrating schematically the portions corresponding to low M_r u-PA (LMW u-PA), the amino-terminal fragment (ATF), the growth factor domain (GF), kringle domain (K), connecting peptide (CN), and catalytic domain (CAT). The numbers refer to residues in the amino acid sequence. Shown beneath the ATF are the specificities of the monoclonal antibodies used and the peptides synthesized, and above the ATF is shown the portion corresponding to the ΔF·u-PA construct.

binding, and it represented a chemically defined sulfated polyanion. The bound u-PA activity was a linear function of the u-PA concentration added to the wells (Figure 3), even at low concentrations of dextran sulfate coatings, so that it was not possible under the conditions of these experiments to study saturation kinetics and derive a binding constant. Nonspecific binding was blocked by pretreatment of dextran sulfate coated wells with 0.5% BSA, and 0.5% BSA was also present during the binding of u-PA. Soluble dextran sulfate inhibited u-PA binding: 50% inhibition of u-PA binding to immobilized dextran sulfate was obtained with only 3 μg of dextran sulfate/mL (not shown). Furthermore, a marked difference was found between the binding to dextran sulfate of the high and low M_r forms of u-PA (Figure 3), which both have catalytic activity and only differ by the 135 amino acid sequence of the ATF region (see Figure 4). Closely similar results were obtained with heparin (Figure 5). This result implied that the ATF region of u-PA contains a binding site for heparin-like sulfated polyanions.

Inhibition of Binding by ΔF·u-PA. Recombinant ΔF·u-PA produced in mouse LB6 cells was used in competition experiments to test if an inactive truncated form of u-PA containing the ATF region could compete with intact u-PA

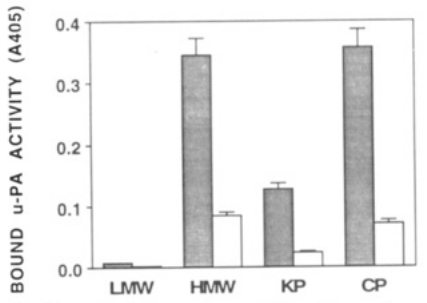

FIGURE 5: Binding of u-PAs to immobilized heparin and effect of synthetic peptides. Microtiter wells were coated with heparin by drying aliquots of a water solution (20 IU/mL; 50 μL/well) in them. The wells were then washed and blocked with a 1:3 dilution of Dulbecco's PBS containing 0.2% BSA. High (HMW) and low (LMW) molecular mass u-PAs (0.5 IU/mL) were then allowed to bind to the wells for 2 h. The effect of the synthetic u-PA kringle peptide (KP) and the control peptide (CP) on high molecular mass u-PA binding was also tested (each peptide at 0.1 mM). The activity of bound u-PA is shown for wells coated with heparin (shaded columns) as well as for uncoated wells (open columns).

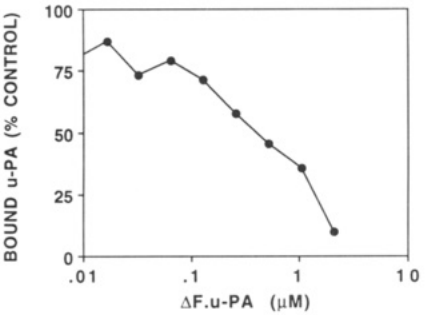

FIGURE 6: Effect of ΔF·u-PA on u-PA binding to immobilized dextran sulfate. Dilutions of ΔF·u-PA in Dulbecco's PBS containing 0.5% BSA and u-PA (2 IU/mL) were allowed to bind to dextran sulfate coated wells for 2 h at 23 °C. The bound u-PA, after rinsing with dilution buffer, was measured by incubation with plasminogen and assay of plasmin thioesterase activity as above. The results are shown as a percentage of the u-PA activity bound in the absence of ΔF·u-PA.

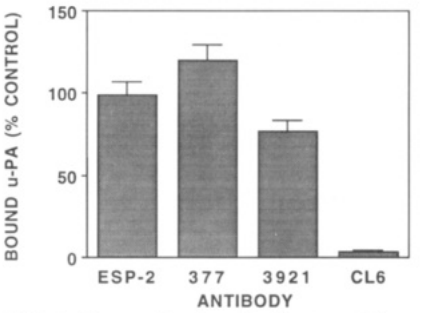

FIGURE 7: Effect of monoclonal antibodies to u-PA on binding of high M_r u-PA to immobilized dextran sulfate. u-PA (2 IU/mL) was preincubated for 2 h at 23 °C with each monoclonal antibody (10 μg/mL) in Dulbecco's PBS containing 0.5% BSA. Aliquots of each preincubation (50 μL) were then applied to dextran sulfate (500 μg/mL) coated microtiter wells and allowed to bind for 2 h. After rinsing, the activity of bound u-PA was assayed as before. The results are shown as a percentage of the u-PA binding obtained with a control preincubation containing no antibody.

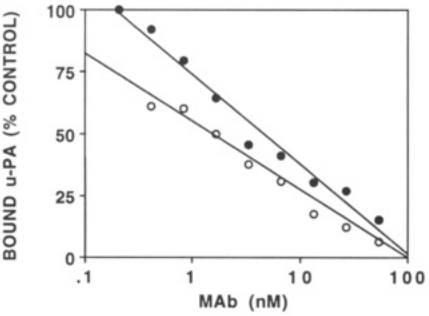

FIGURE 8: Dose effect of monoclonal antibodies with kringle specificity on the binding of u-PA to immobilized dextran sulfate. u-PA (2 IU/mL) was preincubated for 2 h at 23 °C with dilutions of clone 6 (●) and clone 12 (○) monoclonal antibodies in Dulbecco's PBS containing 0.5% BSA. Aliquots (50 μL) of each preincubation were then applied to dextran sulfate (100 μg/mL) coated microtiter wells and allowed to bind for 2 h. After rinsing, the bound u-PA activity was assayed as before. The results are shown as a percentage of the binding obtained with a control preincubation containing no antibody.

for binding to dextran sulfate. ΔF·u-PA (see Figure 4) consisted of residues 1–164 of the human u-PA sequence with an additional 30 amino acid sequence produced by a frameshift (Pedersen et al., 1991). This construct was able to produce a competitive effect on u-PA binding (Figure 6), such that u-PA binding was reduced by 50% in the presence of 9 μg/mL (0.42 μM, representing an 870-fold molar excess) ΔF·u-PA. The competitive effect of this construct was not as strong as may be expected, almost certainly due to the considerable length of irrelevant sequence created by the frameshift.

Inhibition of Binding by Monoclonal Antibodies to u-PA. The involvement of the ATF was therefore further tested using four monoclonal antibodies whose epitopes are known to reside in this region. The differential capacity of these antibodies to bind high M_r u-PA and not low M_r u-PA was clearly evident in binding assays and u-PA bound to any of them retained its enzymatic activity (results not shown). A control monoclonal antibody to t-PA (ESP-2) did not bind either high or low M_r forms of u-PA in the same experiments. When high M_r u-PA was preincubated with these monoclonal antibodies and then applied to dextran sulfate coated wells, strong inhibition of binding was found with monoclonal clone 6, weak inhibition with 3921, and no inhibition with ESP-2 and 377 (actually some promotion) (Figure 7). Since 3921 has specificity for the growth factor domain (supplier's literature), while clone 6 reacts with the kringle domain of u-PA (J. Grøndahl-Hansen, unpublished data), it was evident that binding of u-PA to sulfated polyanions may be mediated by the u-PA kringle structure. Another monoclonal antibody (clone 12) with known kringle specificity (Kobayashi and Grøndahl-Hansen, unpublished) was therefore tested, and it was found that this antibody and clone 6 were both good inhibitors of u-PA binding to dextran sulfate (Figure 8). Clone 12 produced 50% inhibition of u-PA binding with a molar ratio of only 2.8 and clone 6 with a ratio of 11. Clone 12 was also an inhibitor of u-PA binding to heparin (~90% inhibition with a 280-fold molar excess of IgG), while 3921 only produced ~30% inhibition under the same conditions.

Inhibition of Binding by Protamine and Synthetic Peptides. The binding of u-PA in this system was competitively blocked by low concentrations of protamine sulfate, which has also been shown to increase the amount of acid-elutable u-PA recoverable from the surface of RD rhabdomyosarcoma cells (Stephens et al., 1991). Binding of u-PA to dextran sulfate was inhibited 50% by 3 μg/mL (2080-fold molar excess) protamine (Figure 9). Since protamine consists of approximately 60% arginine (Dixon et al., 1986), it appeared likely from this and the above results that the binding of u-PA to sulfated polyanions may involve an electrostatic interaction with basic amino acid residues in the u-PA kringle. A decapeptide (KP) was therefore synthesized corresponding to the inside loop sequence (Steffens et al., 1982) of the human u-PA kringle from Arg-52 to Trp-62, which contains three

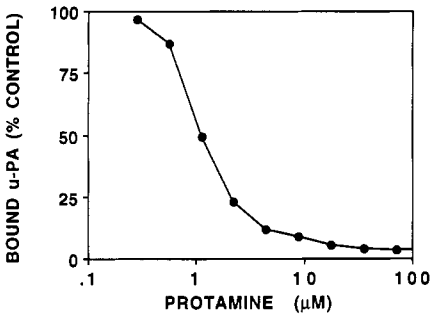

FIGURE 9: Effect of protamine on binding of u-PA to immobilized dextran sulfate. Aliquots (50 μL) of a mixture of u-PA (2 IU/mL) and protamine sulfate dilutions as shown were applied to dextran sulfate (50 μg/mL) coated microtiter wells and allowed to bind for 2 h. After rinsing, the activity of bound u-PA was assayed as before. The results are shown as a percentage of the binding obtained in the absence of protamine.

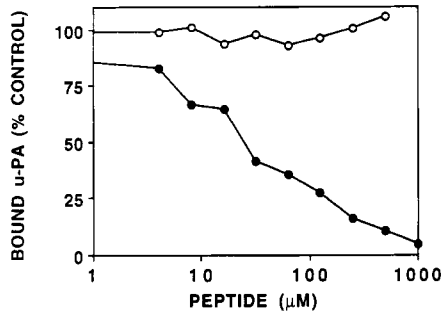

FIGURE 10: Effect of synthetic u-PA peptides on the binding of u-PA to immobilized dextran sulfate. Two synthetic decapeptides were used, KP and CP, derived from the human u-PA amino acid sequence as shown in Table I. Aliquots (50 μL) of a mixture of u-PA (2 IU/mL) and each peptide at the dilutions shown were applied to dextran sulfate (50 μg/mL) coated microtiter wells and allowed to bind for 2 h. After rinsing, the activity of bound u-PA was assayed as before. The results for KP (●) and CP (○) are shown as a percentage of the binding obtained in the absence of peptide.

consecutive arginine residues in positions 57–60 (Figure 2). This peptide was readily soluble in water and could be tested directly for competition with u-PA binding to dextran sulfate. A control decapeptide (CP) covering Lys-136–Lys-145 in the connecting peptide domain of u-PA (see Figure 4) was also synthesized and tested in the same experiments. It was found that the kringle peptide KP inhibited u-PA binding, so that 50% inhibition was obtained at 20 μM, representing a 40 000-fold molar excess (Figure 10). By contrast, the control peptide CP (which was also water soluble) had no significant effect, even at 500 μM (1-million-fold molar excess). The synthetic kringle peptide KP (but not the control peptide, CP) also produced inhibition of u-PA binding to immobilized heparin (Figure 5).

^{1}H NMR Titration of u-PA Kringle by Heparin. The aromatic ^{1}H NMR spectral region of the u-PA kringle was found to be sensitive to the presence of heparin (Figure 11B–D). The aromatic resonances showed an overall broadening of the spectral lines and changes in chemical shifts concomitant with heparin additions. The pH* was monitored before and after adding heparin to verify its constancy throughout the ligand titration experiments. It was noticeable that His resonances where the most affected by heparin. These are indicated by kinked vertical lines in Figure 11. At a [heparin]/[kringle] molar ratio of 0.1 (Figure 11C) shifts were observed for the His ring systems labelled H-I, H-II, and H-III. This pattern repeated itself when the ligand/protein ratio was increased to 1.0 (Figure 11D). However, as the ratio was further increased, the magnitude of the shift plateaued,

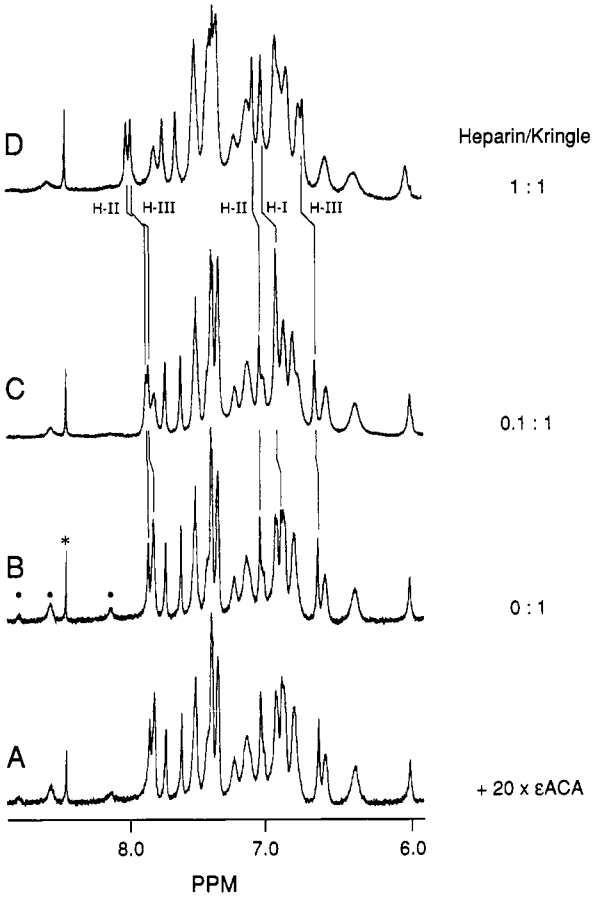

FIGURE 11: ^{1}H NMR aromatic spectra of u-PA kringle in the presence of heparin. (B) Reference spectrum: ligand-free kringle. (C) Kringle in the presence of low M_r heparin, with a molar ratio of [heparin]/[kringle] = 0.1. (D) Same as (C) except that [heparin]/[kringle] = 1.0. (A) Kringle in the presence of a 20-fold molar excess of 6-aminocaproic acid. The shifts induced on selected kringle His resonances are indicated by kinked vertical lines. The u-PA kringle was dissolved in ^2H_2O at 1.5 mM, pH 7.2, and 24 °C. Resonances arising from slowly exchanging amide NH protons are indicated by (•). The asterisk (*) denotes a contaminant signal.

indicating saturation (Figure 12, curve A). It can be seen that the His imidazole H2 resonances underwent larger perturbations than those stemming from the H4 proton on the same ring, a relative effect similar in magnitude and shift direction to what was observed in the course of pH titrations (unpublished). Overall, the H-II and H-III resonances were relatively more perturbed than those from H-I. These resonances have been tentatively assigned to H-48a, H-40, and H-37, respectively, in the kringle sequence.

Heparin Binding Affinity. In an effort to further characterize and to quantify the heparin/kringle interaction, effective binding constants were determined for solutions containing increasing concentrations of NaCl (0.125 and 0.25 M). Titration profiles are shown in Figure 12. From these, estimates of K_a* were derived as described in Materials and Methods. The profile in salt-free solution (Figure 12, curve A) was hyperbolic and yielded K_a* 57.8 mM^{-1}, which indicates a substantial affinity of the u-PA kringle for heparin. When NaCl was added to a concentration of 0.125 M, the binding affinity decreased approximately 3-fold (K_a* 15.9 mM^{-1}) while still exhibiting a heparin titration profile that was similar, at low [heparin], to the previous one (Figure 12, curve B). However, as indicated by the nonsaturation profile at [heparin]/[kringle] > 2, some evidence for nonspecific binding and/or artifacts reflecting the heterogeneity of the ligand was observed. As the NaCl concentration was increased to 0.25

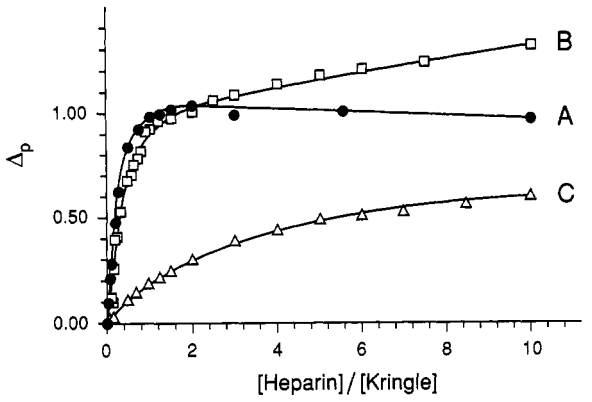

FIGURE 12: Heparin titration profiles of the u-PA kringle. Data points were obtained from ^{1}H NMR experiments under the conditions given for Figure 11. The profiles outline the response of kringle His singlets to heparin additions. Δ_p is the fraction of heparin-bound kringle, $(\delta-\delta_f)/(\delta_b-\delta_f)$, where δ is the observed (exchange-averaged) chemical shift of a kringle resonance at a certain titration point and δ_f and δ_b are the limit chemical shifts of the resonance for ligand-free and ligand-bound kringle, respectively (De Marco et al., 1987). Δ_p is plotted versus [heparin]/[kringle], the ratio of total heparin concentration to total kringle concentration, for salt-free (A), 0.125 M NaCl (B), and 0.25 M NaCl (C) solutions. The continuous curves were computed on the basis of the determined K_a* values (see Materials and Methods). Additional data points recorded at higher [heparin]/[kringle] ratios (used for the K_a* calculations) fall outside the range covered by the figure.

M, the affinity further decreased to K_a* 0.25 mM^{-1}.

Insolubility of the Heparin–Kringle Complex. We noticed in the course of heparin titrations in the absence of salt that the kringle solution (1.5 mM) became turbid. The turbidity disappeared at [heparin]/[kringle] > 1. A similar effect has been observed in investigations of the interaction of heparin with thrombin (Olson et al., 1991). In our study, this effect did not occur at 0.125 or 0.25 M NaCl.

DISCUSSION

Heparin has several effects on u-PA, including stimulation of cell secretion (Falcone, 1989), enhancement of proenzyme activation (Lijnen & Collen, 1986; Watahiki et al., 1989), promotion of activity (Andrade-Gordon & Strickland, 1986; Pâques et al., 1986; Stephens et al., 1991), modulation of inhibition (Stump et al., 1986b) and possibly a contribution to cell/matrix binding (Stephens et al., 1991). Clearly a definition of the structural determinants of the interaction between u-PA and heparin is an important prerequisite for an understanding of these phenomena. From comparative sequence data on heparin-binding proteins (Cardin & Weintraub, 1989), we proposed that the tripeptide Arg-109-Arg-110-Arg-111 within the kringle inner loop is likely to define a key feature of the u-PA heparin-binding site (Stephens et al., 1991). This is now supported by binding studies with high and low M_r forms of u-PA, the use of monoclonal antibodies to the ATF region, and competitive binding assays with synthetic u-PA peptides.

Previously there has been no known biological function for the u-PA kringle region, and the study reported here is the first to establish an interaction with this u-PA structure. The location of the heparin-binding site within the kringle region is consistent with the N-terminal location of heparin-binding sites in tissue-type plasminogen activator (t-PA), which has one in the finger domain and one in kringle 2 (Stein et al., 1989), and also with those in fibronectin (Petersen et al., 1980). It is notable that while u-PA and t-PA both have a growth factor domain in their N-terminal regions and both bind heparin, the respective heparin-binding sites are not located within this domain but in additional elements. This contrasts with observations of a growing number of growth factors which bind to heparin (Thomas & Gimenez-Gallego, 1986).

The turbidity observed in salt-free solution at low [heparin]/[kringle] suggests that more than one kringle unit binds electrostatically to the polyanion at low heparin concentration, thus generating an insoluble aggregate. At high salt, the complexation reaction is less favored and the turbidity effect is avoided. Basic amino acids in the u-PA kringle sequence are implicated in this interaction with heparin. The u-PA kringle has five Lys and seven Arg residues, and in the ^{1}H NMR experiments spectral shifts were noticed for a number of peaks in the aliphatic Lys/Arg region of the kringle 2D-COSY spectrum (not shown); however, these have not yet been assigned. Thus is it is not yet possible to pinpoint precisely which of the kringle basic residues interact with heparin. The sequential assignment of the ^{1}H NMR spectrum (in progress) is expected to clarify this point. However the grouping of three consecutive arginine residues (Arg-57–Arg-60) in the kringle (Figure 2) is a likely component of the binding site. Comparison with the data of Cardin and Weintraub (1989) shows similarity to one grouping of heparin-binding proteins, which have the common motif they designate as -X-B-B-B-X-X-B-X-. The single basic residue (B) position of this motif is occupied by a half-cystine residue in urokinase (Cys-63), so there is not complete identity. However, particularly in a kringle structure, features of the protein folding may be more important than the primary sequential array of basic amino acids in determining suitable clusters of cationic side chains that can act as heparin-binding sites.

For the ^{1}H NMR titration of u-PA kringle with heparin, we monitored the selective perturbation of His imidazole resonances upon heparin binding, a phenomenon which has recently been reported in ^{1}H NMR studies of the interaction of heparin with antithrombin III (Gettins, 1987; Gettins & Wooten, 1987) and with bovine platelet factor 4 (Talpas et al., 1991). These aromatic perturbations suggest that, in addition to cationic aliphatic side chains, the imidazole rings of His residues may be important contributors to the binding of heparin to proteins. It is noteworthy that none of the five His residues of the u-PA kringle occupy sites within the canonical Lys-binding site described for the plasminogen kringles 1, 4, and 5 and for the t-PA kringle 2 (De Marco et al., 1987; Tulinsky et al., 1988; Byeon et al., 1989; Thewes et al., 1990). Hence, it is suggested that the interaction between the u-PA kringle and heparin is likely to involve other regions of the domain. In this context, it is interesting to note that a 20-fold molar excess of 6-aminocaproic acid does not perturb the u-PA kringle spectrum (Figure 11A,B), while 6-aminocaproic acid is known to affect the NMR spectra of all the other kringles mentioned above (Ramesh et al., 1987; Petros et al., 1989; Thewes et al., 1990; Byeon et al., 1991). Since the u-PA kringle exhibits no detectable affinity (K_a* <0.05 mM^{-1}) toward the ω-amino acid ligands, consistent with the fact that u-PA is not retained by lysine-conjugated affinity matrices (Winkler et al., 1985), we conclude that its binding specificity and, in all likelihood, its binding-site structure are different from that of the ω-amino acid binding kringles. We have also tested the sulfonated kringle ligand *p*-benzylaminesulfonic acid (BASA) and were unable to detect an interaction with the u-PA kringle. Thus, it is apparent that the latter differs from plasminogen and t-PA kringles in that while it does not bind small zwitterionic ligands, it does show affinity for heparin fragments.

Low M_r heparin was chosen for the NMR study in order to minimize spectral complexity and overall broadening. However, it is to be expected that the strength of the interaction should increase with the size of the ligand molecular (Luscombe & Holbrook, 1983). Andrade-Gordon and Strickland (1986) have shown that heparin (average M_r ~16 500; Dawson et al., 1986) increases the activity of u-PA. The authors also estimated a K_a ~345 mM^{-1} for the interaction of high M_r heparin with u-PA. Assuming that the number of binding sites exposed by the heparin molecule is proportional to the size of the polymer, for a heparin fragment of average M_r 3000, as used in our study, one would estimate a K_a^* ~62.7 mM^{-1} based on the data for intact heparin, a value which is comparable to the K_a^* ~57.8 mM^{-1} that we measured. Taking into account the slightly different experimental conditions in the two studies, the agreement suggests that most of the affinity of heparin for u-PA can be accounted for by its interaction with the kringle domain. At 0.25 M NaCl, the relatively weak binding observed (K_a^* ~0.25 mM^{-1}) can be attributed to the expected ionic strength shielding effect on the kringle/heparin interaction. However, 0.25 M NaCl amply exceeds the physiological salt concentration.

By interpolating (Stineman, 1980) the data obtained at zero ionic strength and at 0.125 and 0.250 M NaCl, a K_a^* ~12 mM^{-1} was estimated for [NaCl] ~150 mM. The latter salt concentration is close to the physiological ionic strength. This K_a^* is similar in magnitude to the affinities of L-lysine, 6-aminocaproic acid, or fibrinogen for plasminogen kringles 1, 4, and 5, 60 $mM^{-1} < K_a < 0.1$ mM^{-1} (Petros et al., 1989; Thewes et al., 1990; Menhart et al., 1991; Rejante et al., 1991a,b). Similarly, in the case of the t-PA kringle 2, its K_a for L-lysine is ~17.9 mM^{-1} (Byeon, 1991). K_a is equivalent to k_{on}/k_{off}, where k_{on} and k_{off} are the rate constants for association and dissociation. If one assumes, as is the case for other kringles (De Marco et al., 1987; Petros et al., 1989) that k_{on} is diffusion controlled, then it is the k_{off} which determines the affinity of heparin to the u-PA kringle. Physiologically, a low k_{off} (i.e., high K_a) value implies a relatively longer residence time of the u-PA molecular at a fixed locus within the intercellular matrix, which may not be physiologically efficient if the u-PA has ultimately to reach and bind to its cell receptor in order to be functionally active (Rønne et al., 1991).

Low-affinity u-PA kringle interactions with proteoglycans may serve to retain low but significant concentrations of u-PA in the vicinity of cells secreting this enzyme, so that, during subsequent movement of cells through the matrix, formation of new cell contacts will also facilitate loading and concentration of u-PA onto cell receptors, the site of functional activity (Pöllänen et al., 1990; Rønne et al., 1991). An analogous system may be the low-affinity binding of bFGF to glycosaminoglycans before subsequent loading onto high-affinity cellular receptors (Yayon et al., 1991). By this mechanism the localization of u-PA and hence the plasminogen activation system would proceed at the junction of cell and matrix, i.e., focal contacts, where proteolysis of adhesion proteins could then complete the cell attachment/detachment cycle.

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