Lysine-50 is a likely site for anchoring the plasminogen N-terminal peptide to lysine-binding kringles

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(RECEIVED March 12, 1998; ACCEPTED June 15, 1998)

Abstract

Interactions between the kringle 4 (K4) domain of human plasminogen (Pgn) and segments of the N-terminal Glu1–Lys77 peptide (NTP) have been investigated via ¹H-NMR at 500 MHz. NTP peptide stretches devoid of Lys residues but carrying an internal Arg residue show negligible affinity toward K4 (equilibrium association constant $K_a < 0.05 \text{ mM}^{-1}$). In contrast, while most fragments containing an internal Lys residue exhibit affinities comparable to that shown by the blocked Lys derivative N^α-acetyl-L-lysine-methyl ester ($K_a \sim 0.2 \text{ mM}^{-1}$), peptides encompassing Lys50 consistently show higher K_a values. Among the investigated linear peptides, N^α-acetyl-Ala-Phe-Tyr-His-Ser-Ser-Lys50-Glu-Gln-NH₂ (AcAFYHSK50EQ-NH₂) exhibits the strongest interaction with K4 ($K_a \sim 1.4 \text{ mM}^{-1}$), followed by AcYHSK50EQ-NH₂ ($K_a \sim 0.9 \text{ mM}^{-1}$). Relative to the wild-type sequence, mutated hexapeptides exhibit lesser affinity for K4. When a Lys50 → Ser mutation was introduced (⇒ AcYHSS50EQ-NH₂), binding was abolished.

The Ile27–Ile56 construct (L-NTP) contains the Lys50 site within a loop constrained by two cystine bridges. The propensity of recombinant Pgn K1 (rK1) and K2 (rK2) modules, and of Pgn fragments encompassing the intact K4 and K5 domains, for binding L-NTP, was investigated. We find that L-NTP interacts with rK1, rK2, K4, and K5—all lysine-binding kringles—in a fashion that closely mimics what has been observed for the Glu1–HSer57 N-terminal fragment of Pgn (CB-NTP). Thus, both the constellation of kringle lysine binding site (LBS) aromatic residues that are perturbed upon complexation of L-NTP and magnitudes of kringle-L-NTP binding affinities (rK1, $K_a \sim 4.3 \text{ mM}^{-1}$; rK2, $K_a \sim 3.7 \text{ mM}^{-1}$; K4, $K_a \sim 6.4 \text{ mM}^{-1}$; and K5, $K_a \sim 2.1 \text{ mM}^{-1}$) are essentially the same as for the corresponding kringle-CB-NTP pairs. Molecular modeling studies suggest that the Glu39–Lys50 stretch in NTP generates an area that complements, both topologically and electrostatically, the solvent-exposed kringle LBS surface.

Keywords: kringle lysine-binding; plasminogen activation peptide; plasminogen N-terminal peptide; preactivation peptide loop

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Abbreviations: 1D, one-dimensional; 2D, two-dimensional; Ac, acetyl; AcNHCH₂COCH₃, blocking group; CB-NTP, CNBr peptide fragment Glu1–HSer57 from NTP; COSY, 2D chemical shift correlated spectroscopy; HPLC, high-performance liquid chromatography; rK1, recombinant human plasminogen kringle 1 (Tyr80–Glu164); rK2, recombinant human plasminogen kringle 2 (Glu164–Thr244; Cys169 → Gly); rK3, recombinant human plasminogen kringle 3 (Thr253–Ser335; Cys297 → Ser); K4, human plasminogen kringle 4 (Val355–Ala440); K5, human plasminogen kringle 5 (Val454–Phe546); K_a , ligand-kringle equilibrium association constant; LBS, lysine binding site; L-NTP, synthetic Ile27–Ile56 segment of the human plasminogen NTP (C-terminus amidated); NTP, N-terminal (Glu1–Lys77) peptide domain of human plasminogen; pH*, glass electrode pH reading uncorrected for deuterium isotope effect; tPA, tissue-type plasminogen activator; TPPI, time proportional phase incrementation.

It was originally observed (Wiman & Wallén, 1975) that fragments related to the plasminogen (Pgn) N-terminal peptide (NTP, Glu1–Val79), particularly the tryptic heptapeptide Ala-Phe-Gln-Tyr-His-Ser-Lys50 (AFQYHSK50) that originates from the NTP Cys30–Cys50 loop, are retained upon passage through a Pgn-Sepharose column. An interaction between the CNBr-generated fragment Glu1–HSer57 (CB-NTP) and the Pgn kringle 1 (K1) domain was subsequently measured by Rickli and coworkers (Lerch et al., 1980).

In an accompanying paper (An et al., 1998), we present ¹H-NMR spectroscopic evidence indicating that CB-NTP interacts with recombinant K1 (rK1), rK2, K4, and K5, all lysine-binding kringles, with K4 exhibiting a somewhat higher affinity, while rK3, which is devoid of lysine binding capability (Söndel et al., 1996), fails to interact. Based on similar experiments, it was previously demonstrated (Ramesh et al., 1995) that AFQYHSK50,

as well as shorter versions thereof, FQYHSK50, QYHSK50, and YHSK50, bind to the Pgn K4 domain with association constant (K_a) values in the $2.5\text{--}8.5\text{ mM}^{-1}$ range. Although the NMR data confirmed a potential for the AFQYHSK50 segment to mediate the binding between the Pgn NTP and the lysine-binding kringle domains, the doubt subsisted as to the functional significance of these interactions since it is well established that lysine-binding kringles interact preferentially with C-terminal, zwitterionic, Lys residues (Byeon et al., 1995; Marti et al., 1997) while in the intact Glu1-Pgn all basic residues within the NTP domain are internal, i.e., N^α/C' -blocked. Related to this issue is the question whether kringles do at all interact with peptides carrying internal Lys and/or Arg residue(s) as it is well known that small ligands, such as AcLysOMe and AcArgOMe, which mimic such compounds, bind to Pgn kringles with relatively low affinity: in the case of K4, with $K_a \sim 0.2\text{ mM}^{-1}$ (Fig. 1C) and 0.08 mM^{-1} , respectively (Rejante et al., 1991). Therefore, a lingering question is whether in the intact Glu1-Pgn there are Lys- or Arg-containing sequences within the NTP capable of mediating intramolecular interaction with kringle(s) with a reasonable degree of specificity. This is directly related to the bigger question: "How do proteins, which do not contain free lysine, bind to plasminogen?" (Hochschwender & Laursen, 1981).

It may be argued that the conformation of the NTP, particularly around the doubly bridged (Cys30–Cys54/Cys34–Cys42) loop that contains the AFQYHSK50 stretch, might expose side-chain carboxylate groups, which would confer dipolar character to the area encompassing the Lys50 cationic center. Indeed, indirect evidence suggests that the contiguous Glu51 residue (Urano et al., 1991; Takada et al. 1993) could stabilize binding through electrostatic interactions with exposed cationic loci at the kringle lysine-binding site (LBS). Alternatively, we have speculated that the more removed Glu-Glu-Asp-Glu-Glu39 stretch within the NTP might participate in such a role (Ramesh et al., 1995).

In this paper, we address the above questions via a systematic search for potential interactive loci within the NTP. By ^1H -NMR spectroscopy we have screened short synthetic peptides that duplicate NTP sequences containing internal Lys and Arg residues, for complexation to the Pgn K4 domain. As described below, we find that linear peptide sequences of NTP encompassing Lys50 exhibit the most marked association to K4. Among the investigated linear peptide ligands, we identify AcAFQYHSK50EQ-NH₂, a nanopptide that incorporates the previously identified activation heptapeptide sequence (Wiman & Wallén, 1975), as the one that exhibits the highest affinity ($K_a \sim 1.4\text{ mM}^{-1}$). Furthermore, we

Fig. 1. ^1H -NMR COSY spectra of plasminogen kringle 4: ligand perturbation of aromatic resonances. Ligands are (A) AcLysOMe, (B) AcAFQYHSK50EQ-NH₂, (C) L-NTP, and (D) CB-NTP. Numbers in parenthesis refer to the peptide code number, as indicated in Table 1. Spectra of ligand-complexed K4 (dark-trace) are superimposed on those of the ligand-free K4 (light-trace). Cross-peak shifts are denoted by curved arrows. Phase-sensitive spectra were recorded on $\sim 0.7\text{ mM}$ kringle samples dissolved in $^2\text{H}_2\text{O}$, [ligand]/[kringle] $\sim 4\text{--}60$.

detect an even stronger binding interaction between a synthetic Ile27–Ile56 construct (L-NTP) that contains the doubly looped Cys30–Cys54 structure, and the individual rK1, rK2, K4, and K5 modules. Finally, we also demonstrate that the affinities of L-NTP for complexation to the Pgn kringle domains are comparable to those determined for CB-NTP, thus establishing the Cys30–Cys54 loop that encompasses the Lys50 site as the key structure that mediates the interaction. The structural implications are discussed on the basis of a molecular model, generated by docking CB-NTP to the K4 LBS, that qualitatively accounts for the main ^1H NMR spectral shifts that result from the domain–domain interactions.

Results

As reported (An et al., 1998), the Glu1–HSer57 fragment of NTP (CB-NTP) interacts with the Pgn rK1, rK2, K4, and K5 domains. The aromatic LBS residues, Phe36, Trp25, Trp62, Phe/Tyr64, Trp72, and Tyr74 (kringle residue numbering convention), are consistently perturbed by ligand complexation and their ^1H -NMR chemical shifts can be monitored to detect the interaction. As ranked by binding affinity, CB-NTP interacts with K4 ($K_a \sim 6.2 \text{ mM}^{-1}$) > rK1 ($K_a \sim 4.6 \text{ mM}^{-1}$) > rK2 ($K_a \sim 3.3 \text{ mM}^{-1}$) > K5 ($K_a \sim 2.3 \text{ mM}^{-1}$) (An et al., 1998). Hence, we have selected K4 for further binding studies centered on synthetic, lysine-containing peptides that replicate segments within NTP. The investigated peptides were either N-acylated or both N-acylated and C-amidated to avoid complications arising from potential electrostatic interactions between peptides' N- and C-termini groups and the K4 ligand-binding site (LBS).

Peptides with Lys50 C-terminus

Previous studies (De Marco et al., 1987) have shown that, relative to other ligands, K4 exhibits a good affinity toward N $^\alpha$ -acetyl-L-Lys (AcLys) ($K_a \sim 37 \text{ mM}^{-1}$), a low MW model compound that mimics a C-terminal Lys residue. Evidence has also been reported that the Pgn K4 (Ryan & Keegan, 1985; Balciunas et al., 1993; Ramesh et al., 1995) and tPA K2 (Byeon et al. 1995) bind oligopeptides with Lys C-terminus. We have now tested the Lys C-terminus heptapeptide AcAFQYHSK50 (1), as well as the shorter derivatives AcFQYHSK50 (2), AcQYHSK50 (3), and AcYHSK50 (4), as ligands for the human Pgn kringle 4 (K4) domain. Except for acylation of the N-terminus, these peptides are identical to, or derived from, the AFQYHSK50 activation heptapeptide (Wiman & Wallén, 1975), previously investigated by ^1H -NMR (Ramesh et al., 1995). COSY aromatic spectra of K4 indicate that resonances from Trp25, Trp62, Trp72—residues all located at the LBS—are essentially identically perturbed by both AcAFQYHSK and AcLys (not shown). From the ligand-induced chemical shifts, binding profiles can be obtained and K_a values estimated via numerical fitting the ligand titration isotherms. For peptides (1)–(4), the K_a values are: $\sim 3.8 \text{ mM}^{-1}$ (1), $\sim 17.2 \text{ mM}^{-1}$ (2), $\sim 2.7 \text{ mM}^{-1}$ (3), $\sim 6.2 \text{ mM}^{-1}$ (4). By reference to reported results (Ramesh et al., 1995), the K_a 's indicate that acylation of the N-terminus is of little consequence for the binding, except for an enhancement of affinity suggested for peptide (2). In view of the preference of lysine-binding kringles for N $^\alpha$ -blocked Lys residues, it is not surprising that these peptides interact with the Pgn K4. However, relative to the model compound AcLys ($K_a \sim 37 \text{ mM}^{-1}$), the interactions are significantly weak ($K_a < 18 \text{ mM}^{-1}$). Hence, by comparison to AcLys, peptides (1)–(4) cannot be considered to afford conclusive

evidence to justify ascribing selective biochemical functionality to the AFQYHSK50 sequence. Further experiments discussed below show that this caveat is unwarranted.

Peptides containing internal lysine residues

Binding to K4 of N-acylated/C-amidated peptides related to the AFQYHSK50 sequence was investigated: AcSK50EQ-NH $_2$ (5), AcHSK50EQ-NH $_2$ (6), AcYHSK50EQ-NH $_2$ (7), and AcAFQYHSK50EQ-NH $_2$ (8). Relative to (1)–(4), these peptides extend the C-terminus two residues beyond Lys50. Panel B in Figure 2 illustrates COSY spectra of K4 $\pm$ peptide (8); as revealed by this experiment, and consistent with what is observed for peptides (5), (6), and 7 (not shown), these peptides interact with the kringle LBS residues in a fashion similar to that indicated in Figure 1A for AcLysOMe.

From the NMR ligand binding profiles (not shown) we note that, while the shorter peptides (5) and (6) bind K4 in a fashion comparable to AcLysOMe ($K_a \sim 0.2 \text{ mM}^{-1}$), peptides (7) and (8) interact with K4 with higher affinity ($K_a \sim 0.9$ and 1.4 mM^{-1} , respectively). Considering that in (7) and (8) Lys50 is not C-terminus, and in view of the weak affinity of AcLysOMe for K4, these results afford strong evidence supporting a Lys50-containing segment within the NTP as mediating the interaction of the latter with kringle domains in the intact Glu1–Pgn. The data also indicate that besides the Lys50 restricted locus, there are other factors that determine the peptides' affinities for K4, as residues preceding His48, Tyr47 in particular, contribute positively to the binding interaction.

Other N-acylated/C-amidated NTP sequences were investigated: AcVTKK20QLG-NH $_2$ (9), AcAAK33C(X)E-NH $_2$ (10), AcENRK62SS-NH $_2$ (11), and AcFEKK78VY-NH $_2$ (12). COSY spectra reveal that these peptides all interact with aromatic residues at the K4 LBS in a fashion essentially identical to that exhibited by AcLysOMe (Fig. 1A) and, from ligand titration plots, $K_a \sim 0.2 \text{ mM}^{-1}$ is obtained for peptides (10)–(12). Interestingly, (9) binds K4 with a relatively higher affinity ($K_a \sim 0.5 \text{ mM}^{-1}$), reflecting a favorable interaction with the Lys–Lys20 sequence. In contrast, the relatively lower affinity of peptide (12), which also carries a Lys–Lys sequence, could result from a negative electrostatic contribution of the Glu76 side chain.

Peptides containing internal Arg residue(s)

It has been reported (Horrevoets et al., 1995) that mutations, R61A, K62A, R68A, R70A, K77H, K78H, in the “hinge region” that connects the NTP to K1 lead to conformational changes in Pgn. As described above, AcENRK62SS-NH $_2$ (11) exhibits an affinity for K4 comparable to that of AcLysOMe ($K_a \sim 0.2 \text{ mM}^{-1}$) or to NTP segments containing internal Lys residues other than Lys50. Although previous studies (Rejante et al., 1991) have shown that, relative to the Lys-containing analogs, AcArg ($K_a \sim 0.2 \text{ mM}^{-1}$), AcArgOMe ($K_a \sim 0.08 \text{ mM}^{-1}$), and ArgOMe (no binding) do not interact significantly with K4, with the aim of ascertaining the potential role of Arg-containing segments within NTP we have also screened for interactions with K4 of the Arg-containing NTP peptide segments AcEEFTC(X)R43AF-NH $_2$ (13) and AcIIRMR70DV-NH $_2$ (14). The same K4 LBS aromatic resonances responsive to binding of Lys peptides were monitored, at comparable ligand levels. We found that the interaction of K4 with (13) is marginal ($K_a \sim 0.05 \text{ mM}^{-1}$) and with (14) nil. Hence, we con-

Fig. 2. ^1H -NMR shifts of representative kringle LBS aromatic signals resulting from CB-NTP (black-bars) and L-NTP (white-bars) binding. (A) rK1, (B) rK2, (C) K4, and (D) K5. Data extracted from COSY spectra, as illustrated in Figure 1 for K4, or reported in the accompanying paper (CB-NTP) (An et al., 1998).

clude that Arg-containing stretches within the Pgn NTP are unlikely to mediate association of the NTP to lysine binding kringles.

Thus, a distinct range of affinities of NTP-derived peptides for K4, ranking Glu1-HSer57 (CB-NTP) ($K_a \sim 6.2 \text{ mM}^{-1}$) > AcAFQYHSK50EQ-NH₂ ($K_a \sim 1.4 \text{ mM}^{-1}$) > AcYHSK50EQ-NH₂ ($K_a \sim 0.9 \text{ mM}^{-1}$) has been established, which points to the Lys50 stretch as a focal area for the NTP-kringle interaction.

Loop segment Ile27-Ile56: L-NTP

As discussed above, AcHSK50EQ-NH₂ (6) and AcSK50EQ-NH₂ (5) interact with K4 with relatively low affinity ($K_a \sim 0.2 \text{ mM}^{-1}$). If larger polypeptide segments encompassing Lys50 are assumed to contain the requisite residues that afford specificity to the NTP, one would expect that the main conformational constraints that encode for the binding specificity be localized within the Cys30-Cys54 loop.

L-NTP binding to K4 was investigated. K4 COSY spectra (Fig. 1C) reveal that the interaction is qualitatively similar to that of the same kringle with AcLysOMe, AcAFQYHSK50EQ-NH₂, and CB-NTP (Fig. 1A,B,D). We observe that L-NTP binding does not perturb the Tyr2 ring of K4, which is structurally removed from the LBS (Fig. 1C), while the K4 Trp25, Trp62, and Trp72 resonances are affected by L-NTP, essentially replicating the shifts detected upon K4 binding any of the peptides that contain an internal Lys residue.

Within L-NTP, another Lys residue is found occupying site 33. As discussed above, the peptide AcAAK33CE-NH₂ (10) does not interact significantly with K4. To check for potential extended sequence effects on the interaction, the nanopptide AcEEC(X)AAK33C(X)EE-NH₂ (15), which corresponds to the Glu28-Glu36 segment within NTP, was also tested: $K_a \sim 0.2 \text{ mM}^{-1}$ was measured, i.e., the same affinity for K4 as determined for (10). Based on these results, we conclude that Lys33 within the L-NTP is unlikely to mediate the interaction of NTP with the Pgn K4, hence to other kringles.

L-NTP binding to kringle 1, kringle 2, and kringle 5

The homologous lysine-binding kringles of Pgn were also tested for L-NTP binding. LBS aromatic resonance shifts experienced by K1, K2, K4, and K5 resulting from CB-NTP and L-NTP binding are summarized in Figure 2. Bars of positive and negative amplitudes represent CB-NTP and L-NTP induced low- and high-field shifts, respectively. The comparative data support the interpretation that L-NTP and CB-NTP interact with the same set of aromatic residues in each kringle.

From L-NTP binding curves for rK1, rK2, K4, and K5 we determine $K_a \sim 6.4 \text{ mM}^{-1}$ for K4, comparable to $K_a \sim 6.2 \text{ mM}^{-1}$ determined for the larger CB-NTP fragment. Consistently, we also find that rK1 ($K_a \sim 4.3 \text{ mM}^{-1}$), rK2 ($K_a \sim 3.7 \text{ mM}^{-1}$), and K5 ($K_a \sim 2.1 \text{ mM}^{-1}$) all yield affinities that closely match those determined for the association of the same kringles to CB-NTP: rK1 ($K_a \sim 4.6 \text{ mM}^{-1}$), rK2 ($K_a \sim 3.3 \text{ mM}^{-1}$), and K5 ($K_a \sim 2.3 \text{ mM}^{-1}$) (An et al., 1998). The kringle binding interactions of L-NTP are also of higher affinity than those measured for the N α -blocked, C'-blocked Arg or Lys ligands AcLysOMe ($K_a < 0.3 \text{ mM}^{-1}$) and AcArgOMe ($K_a < 0.3 \text{ mM}^{-1}$) (Marti et al., 1997), supporting structural factor(s) within L-NTP as defining the affinity of the Lys50 segment for the lysine-binding Pgn kringles.

Hexapeptide variants

To further substantiate the proposed role of Lys50 and neighboring residues in the binding interaction with K4, we have also investigated the synthetic hexapeptide variants of AcYHSK50EQ-NH₂ (7) ($K_a \sim 0.9 \text{ mM}^{-1}$): AcYHSS50EQ-NH₂ (16), AcYHSK50SQ-NH₂ (17), and AcYHSE50KQ-NH₂ (18) (mutations underlined). From ligand titration experiments on K4, the Lys50 $\rightarrow$ Ser substitution in (16) was found to abolish the binding, confirming the strict requirement of Lys50 for the interaction. Since the peptide contains Tyr47, the result indicates that whatever the contribution of this residue to the interdomain affinity, by itself Tyr47 is insufficient to generate association to K4. A decreased binding ($K_a \sim 0.5 \text{ mM}^{-1}$) relative to wild type (7) ($K_a \sim 0.9 \text{ mM}^{-1}$) was measured for the Glu51 $\rightarrow$ Ser mutant (17). This is consistent with Glu51 contributing to the binding interaction. Finally, the double mutation Ser49 $\rightarrow$ Lys, Lys50 $\rightarrow$ Ser that reverses the sequence of the Ser49-Lys50 stretch (18), was observed to result also in a decreased affinity ($K_a \sim 0.5 \text{ mM}^{-1}$) relative to (7). Taking into account the extra affinity of L-NTP ($K_a \sim 6.4 \text{ mM}^{-1}$) relative to AcAFQYHSK50EQ-NH₂ ($K_a \sim 1.38 \text{ mM}^{-1}$), these results indicate that the L-NTP Cys30-Cys54 loop encodes for specific structural—implying sequence and folding—features that endow the AFQYHSK50EQ stretch with an enhanced affinity for Pgn kringles.

Perturbed resonances of CB-NTP and L-NTP

COSY spectra (not shown) indicate that the interaction between rK1, rK2, K4, and K5 and CB-NTP or L-NTP causes shifts of Phe40 and Tyr47 aromatic resonances of the polypeptide ligands. The patterns of shifts resulting from either CB-NTP or L-NTP binding to the various kringles are essentially superimposable, thus revealing that (1) the two NTP fragments bind to lysine-binding kringles in closely similar fashions, (2) within NTP, the segment that interacts with rK1 is the one that coincides with the L-NTP substructure. These data further support our interpretation that the F40TCRAFQY47HSK50 stretch is involved in the binding, and suggest that aromatic residues at positions 4 and 11 upstream from Lys50, are affected by the interaction.

Discussion

The various kringles' LBSs are thought to be involved in the intermolecular interaction with α_2 -antiplasmin (Wiman et al., 1989) and intramolecularly with the Pgn NTP (Sjöholm et al., 1973; Wallén, 1980; Ramesh et al., 1995). Current paradigm (Wiman & Collen, 1978; Markus, 1996) attributes key roles to these two interactions in the hemostatic regulation of Pgn. However, in contrast to α_2 -antiplasmin, which carries a C-terminal Lys residue, the intact, Glu1-Pgn NTP domain does not satisfy this condition.

In an attempt to elucidate the intramolecular mechanism that controls the conformation of Glu1-Pgn, we have investigated the affinity of the K4 domain for linear NTP peptide segments. With the aim of identifying likely sites involved in the interaction, and characterizing sequence specific effects, synthetic peptides that replicate segments within NTP that contain internal Lys and/or Arg residues, as well as variant versions of Lys50 peptides with altered occupancy of sites 49, 50, and 51, were tested for binding (Table 1). Shifts of K4 aromatic resonances in the presence of the synthetic linear peptide ligands reveal perturbations of key hydro-

Table 1. Affinities of investigated NTP-related peptide sequences for binding to plasminogen kringle 4

NTP peptides		K_a (mM ⁻¹) ^a
(1)	AcAFQYHSK50	3.77 ± 0.43
(2)	AcFQYHSK50	17.16 ± 1.06
(3)	AcQYHSK50	2.68 ± 0.29
(4)	AcYHSK50	6.16 ± 0.54
(5)	AcSK50EQ-NH ₂	0.23 ± 0.05
(6)	AcHSK50EQ-NH ₂	0.18 ± 0.04
(7)	AcYHSK50EQ-NH ₂	0.88 ± 0.11
(8)	AcAFQYHSK50EQ-NH ₂	1.38 ± 0.21
(9)	AcVTKK20QLG-NH ₂	0.45 ± 0.11
(10)	AcAAK33C(X)E-NH ₂ ^b	0.24 ± 0.04
(11)	AcENRK62SS-NH ₂	0.18 ± 0.05
(12)	AcFEKK78VY-NH ₂	0.20 ± 0.05
(13)	AcEEFTC(X)R43AF-NH ₂ ^b	0.05 ± 0.02
(14)	AcIIRMR72DV-NH ₂	nb ^c
(15)	AcEEC(X)AAK33C(X)EE-NH ₂ ^b	0.22 ± 0.03
(16)	AcYHSS50EQ-NH ₂ ^d	nb ^c
(17)	AcYHSK50SQ-NH ₂ ^d	0.45 ± 0.10
(18)	AcYHSE50KQ-NH ₂ ^d	0.52 ± 0.09
(19)	AcCRAFQYHSK50EQQC-NH ₂ ^e	0.57 ± 0.03

^aUncertainties (±) represent average deviation of no less than five measurements, each resulting from monitoring a single K4 LBS resonance in the course of a ligand titration experiment.

^bX, acetamidomethyl (-CH₂-NH-CO-CH₃) blocking group, used to protect the Cys mercapto group.

^cNo detectable binding.

^dMutated residues are denoted with double underline.

^eCyclized via a Cys42–Cys54 disulfide bond.

phobic side chains at the kringle LBS, which closely replicate those observed (An et al., 1998) for the Glu1–HSer57 NTP fragment (CB-NTP). Qualitatively, as well as quantitatively, this is in line with ¹H-NMR binding data on K4 complexes with small molecule model ligands, particularly AcLys and AcLysOMe. The relative strength of the binding interactions, as assessed from the K_a values (Table 1), leads us to propose that NTP peptide stretches containing internal Lys, rather than Arg, residues are preferentially bound by K4.

We have extended the investigation to the doubly S-S bridged Ile27–Ile56 construct (L-NTP) and to all the Pgn lysine-binding kringles rK1, rK2, K4, and K5.⁴ Interestingly, basically the same sets of K4 residues affected by the lysine-containing NTP oligopeptides discussed above are perturbed by L-NTP. Among the lysine-binding kringles, K4 exhibits the highest affinity toward L-NTP ($K_a \sim 6.4$ mM⁻¹), in line with its noted preference for CB-NTP (An et al., 1998). Furthermore, the affinities of Pgn rK1, rK3, and K5 for L-NTP closely match those determined for CB-NTP (Table 2). This evidence, combined with the excellent correspondence between the NMR chemical shift perturbations resulting from CB-NTP and L-NTP binding (Figs. 1C,D, 2), lends strong support to the hypothesis that the NTP interactive locus is within the Cys30–Cys54 loop domain.

⁴The Pgn rK3 and urokinase kringle, which exhibit no lysine binding capability, previously were found not to interact with CB-NTP (An et al., 1998) and therefore were not tested against L-NTP.

Table 2. Affinities of CB-NTP and L-NTP for binding to plasminogen kringles

Kringle	K_a (mM ⁻¹) ^a	
	CB-NTP	L-NTP
rK1	4.6 ± 0.7	4.3 ± 0.5
rK2	3.3 ± 0.5	3.7 ± 0.4
rK3	nb ^b	—
K4	6.2 ± 0.4	6.4 ± 0.4
K5	2.3 ± 0.4	2.1 ± 0.4

^aA dash (—) is used to indicate that a K_a value was not measured. Uncertainties (±) represent average deviation of no less than five independent measurements, each resulting from monitoring a single kringle LBS resonance in the course of a ligand titration experiment.

^bNo detectable interaction with conserved LBS residues.

Two basic residues within the L-NTP, Lys33 and Lys50, are potentially capable of providing the requisite anchoring group. Peptides replicating the Lys33 sequence, AcAAK33C(X)E-NH₂ (10) and AcEEC(X)AAK33C(X)EE-NH₂ (15), were found not to bind to K4 significantly, exhibiting interactions of similar affinity to those determined for AcLysOMe ($K_a \sim 0.2$ mM⁻¹) and other internal Lys-containing NTP segments alien to the Cys30–Cys54 loop (Table 1). In contrast, AcYHSK50EQ-NH₂ (7) and AcAFQYHSK50EQ-NH₂ (8) bind K4 with $K_a \sim 0.88$ mM⁻¹ and $K_a \sim 1.38$ mM⁻¹, respectively, the interaction being abolished by a K50 → Ser substitution, as revealed by mutant peptide (16).

The affinities of AcSK50EQ-NH₂ (5) and AcHSK50EQ-NH₂ (6), which replicate more restricted copies of the local Lys50 sequence, turn out to be low ($K_a \sim 0.2$ mM⁻¹), essentially the same as for the other investigated non-Lys50 peptides that carry internal Lys residues. However, when Tyr47 is present, as in peptides (7) and (8), the K_a increases. Thus, it is suggested that Tyr47 positively contributes to the NTP-kringle binding interaction in a rather specific manner. The mutant peptides, AcYHSK50SQ-NH₂ (17) and AcYHSE50KQ-NH₂ (18), yield $K_a \sim 0.45$ and 0.52 mM⁻¹, respectively, lower than $K_a \sim 0.88$ mM⁻¹ for the wild-type sequence AcYHSK50EQ-NH₂ (7), revealing that the string of residues encompassing the Lys50 site is important for the interaction, to the extent that reversing the relative amino acid ordering of the Lys50–Glu51 stretch to Glu50–Lys51 weakens the binding to K4.

To further test structural constraints on the binding interaction, we have investigated the synthetic cyclic peptide (19) (Table 1), which encompasses the intact tridecapeptide sequence between Cys42 and Cys54 and thus represents a larger Lys50-containing stretch than any of the Lys50-linear peptides discussed above. Contrary to expectations, this disulfide-bridged, structurally restricted peptide binds K4 with $K_a \sim 0.6$ mM⁻¹, i.e., with about half the affinity of the flexible, shorter linear construct (8). Hence, it must be concluded that the native conformational constraints encoded by the intact Cys30–Cys54 sequence, restricted by two cystine bridges and thus conformationally better defined than the rest of the investigated CB-NTP fragment (An et al., 1998), determine the affinity of L-NTP for the K4 binding site ($K_a \sim 6.4$ mM⁻¹).

The effects of short, NTP-derived synthetic linear peptides on the kinetics of tPA/uPA-catalyzed Pgn activation have been tested previously (Takada et al., 1993). While AFQYHS49 was found to

have no effect on the conversion rate, the activation rate was increased by peptides **KK**, **AFQYHSK50** and **AFQYHSK50E**. A plausible explanation of these effects is that competitive binding, by which the peptide segments displace the intact NTP from the kringles' LBSs, causes changes in the Glu1-Pgn conformation thus affecting the rate of Pgn activation. The Japanese investigators further identified the Ala44–Glu51 segment as responsible for the interaction. In this context, we have observed that replacing the Glu51 residue, as in mutant peptide (17), indeed weakens the interaction with K4 (Table 1). However, although a role for Glu51 is suggested, the undetectable binding of mutant peptide **AcYHSS50EQ-NH₂** (16) implies that neither Tyr47 nor Glu51, by themselves, are sufficient to elicit binding.

We thus conclude that electrostatic forces between Lys50 at the NTP and Asp55 + Asp57 in the kringle LBS are the primary factor that determines the K4-NTP binding affinity and that hydrophobic interactions mediated by the NTP Tyr47 side chain contribute to the specificity. For the other investigated peptides containing internal Lys residue(s), we propose that their interactions with K4 are weakened by their lacking an aromatic residue at position 4 relative to the Lys site(s). In the case of the tPA K2, the peptide **YLLK**, from the fibrinogen B β -chain, also interacts with the K2 LBS with a relatively high affinity ($K_d \sim 37.3 \text{ mM}^{-1}$) (Byeon et al., 1995). This is consistent with previous studies showing preferential binding to the Pgn K1 of C-terminus Lys peptides containing an aromatic residue at position 4 (Rajan, 1987).

Mammalian Pdns of known primary sequence, namely macaca (Tomlinson et al., 1989), porcine (Marti et al., 1985; Schaller et al., 1987), bovine (Schaller et al., 1985; Berglund et al., 1995), murine (Degen et al., 1990), and hedgehog (Lawn et al., 1995; Belczewski et al., 1996), exhibit 65–92% interdomain identity to the human (Forsgren et al., 1987) NTP (Fig. 3). Most substitutions seem to occur within the Phe15–Glu29, Glu36–Glu39, and Arg61–Ile67 stretches, the latter within the “hinge region.” The most conserved residues or stretches are Leu3–Ser16, Cys30–Glu35, Phe40, Cys42–Asn60, and Arg68–Lys78. If one assumes that the Lys50-mediated binding interactions of NTP with kringles is retained among the species variants, then the overall global folding of the corresponding NTP binding domains ought to be similar to that of the human CB-NTP. In particular, conservancy of the **FQYHSK50** segment, which contains Phe45, Tyr47, and Lys50, implies an important role for the “activation heptapeptide” of Wiman and Wallén in determining the Pgn structure and function. In this context, it is suggestive that the NTP-variant genes PRG-A and PRG-B, whose DNA has been found in human cancer cells, encode for this sequence as well (Ichinose, 1992; Weissbach & Treadwell, 1992).

An attempt was made to dock CB-NTP to the K4 LBS, starting from the reported solution structures of horse Pgn K4 (Cox et al.,

1994) and CB-NTP (An et al., 1998). The guiding principle was that the NTP Lys50 side chain electrostatically interacts with the Asp55 and Asp57 carboxylate groups at the K4 LBS. Initially, CB-NTP was manually positioned in proximity to the K4 LBS, with the NTP Lys50 ϵ -amino group pointing toward the K4 Asp55 + Asp57 anionic site. In a second step, energy minimization was implemented under conventional molecular mechanics force fields that incorporate van der Waals and Coulombic interactions (see Materials and methods). NTP residues 44–51 were left unrestricted to move and interact with K4 while the latter was kept rigid. This led to favorable contacts between the NTP Tyr47 and the K4 Trp62 and Trp72, key hydrophobic residues at the kringle LBS. Also, a possible surface of interaction between Phe40 of NTP and the side chain of K4 residue 32, Trp32 in case of the horse Pgn K4, was indicated. This matches the experimental observation that resonances of Phe40 and Tyr47 shift upon binding NTP to the various kringles. The resulting model for the complex is shown in Figure 4B. Figure 4A is identical to Figure 4B except that, to facilitate visualization, the two molecules are shown with 10 Å separation. Figure 4C illustrates side chain contacts at the domain–domain interface.

Inspection of the model reveals that the Tyr47 ring could fit within the groove defined by the Trp62 and Trp72 indole rings: average C–C distances for Trp62 (K4)–Tyr47 (CB-NTP), and Trp72 (K4)–Tyr47 (CB-NTP) are ~ 6.2 and ~ 5.5 Å, respectively. Thus, the model assists to visualize the stabilizing role of the aromatic residue at position –4 upstream from Lys50. The model also enables us to estimate the suggested $\gamma\text{COO}^- \cdots \delta\text{NH}_3^+$ electrostatic pairing distances between K4 and the ligand: ~ 5.9 Å for Asp55 (K4)–Lys50 (CB-NTP) and ~ 3.1 Å for Asp57 (K4)–Lys50 (CB-NTP). Although the $\text{O} \cdots \text{N}$ distance between the Glu39 γCOO^- of NTP and Arg71 δN of K4 is rather large (~ 6.6 Å), taking into account the marked segmental flexibility of the NTP (whereas most of the peptide backbone was kept rigid during docking), the possibility suggests itself of a significant electrostatic interaction between the polyanionic EEDEE39 segment of NTP and K4. It is our expectation that future structural studies on NTP-kringle complexes will help to improve the approximate representations depicted in Figure 4.

Materials and methods

Chemical and biochemical procedures

Protein samples, rK1, rK2, K4, K5, CB-NTP, and L-NTP, belonged to batches described elsewhere (An et al., 1998). Other NTP peptide fragments were synthesized via a chemistry similar to that used to generate the Ile26–Ile56 (L-NTP) fragment (An et al.,

Fig. 3. Homology alignment of plasminogen NTP (Glu1–Val79) sequence variants from various species. Also included are two human Pgn-related gene proteins, PRG-A and PRG-B (Ichinose, 1992; Weissbach & Treadwell, 1992). The strictly conserved **FQYHSK50** sequence is highlighted by shadowed background.

Fig. 4. Computer generated docking of CB-NTP to Pgn kringle 4 (**A,B**). The molecular model shows in space-filling (van der Waals) representation the CB-NTP in magenta and the K4 in blue. A magnified view of the interface contact area between NTP segment Phe40–Lys50 and K4 residues Trp32, Asp55, and Asp57 is shown in stick diagram representation (**C**). The graphics were generated from the NMR solution structures of the horse Pgn K4 (Cox et al., 1994) and human CB-NTP (An et al., 1998), with Quanta 4.3 software (Molecular Simulations, Inc., San Diego, California).

1998). Parallel peptide chain assemblies were carried out on an Abimed AMS 422 multiple peptide synthesizer using a 9-fluorenylmethoxycarbonyl/tert-butyl (Fmoc/tBu) strategy: Fmoc group removal with piperidine-N,N-dimethylformamide (DMF) (2:8) (2 × 10 min), DMF washings (6 × 0.2 min), amino

acid couplings (45 min), and DMF washings (6 × 0.2 min). Deprotection and coupling times were increased as the synthesis progressed. For the coupling, solutions of Fmoc-amino acids (4 eq, 0.7 M), N-[(¹H-benzotriazol-1-yl)(dimethylamino) methylene]-N-methylmethanaminium tetrafluoroborate N-oxide (TBTU, 4 eq, 0.45 M), N-methyl-morpholine (NMM, 8 eq, 0.9 M) were sequentially added to the peptide-resins. All peptides were blocked at the N-terminus with acetyl group. The C-termini of all peptides were in form of amide, except four C-terminal Lys peptides (1)–(4). For the amide peptides a Fmoc-5-(4 eq*), N-[(dimethylamino) (¹H-1,2,3-triazolo[4,5-*b*]pyridin-1-yl) methylene]-N-methyl methanaminium hexafluorophosphate N-oxide (HATU, 4 eq*) and 7-aza-1-hydroxybenzotriazole (HOAt, 4 eq*) were dissolved to a final concentration of 0.3 M with a solution of N,N-diisopropylethylamine (DIEA, 0.6 M) in DMF and (PAL-PEG-PS)-resin was used. The free acid peptides were synthesized on a *p*-alcoxybenzyl handle polyethylene glycol-polystyrene (AB-PEG-PS)-resin with the residue of Lys incorporated. The Thiol group of side-chain Cys was permanently blocked with the acetamidomethyl (Acm) group, when required. This group is completely stable to all protocols followed for the synthesis, including TFA cleavage. For the synthesis of the cyclic peptide (Cys42–Cys54)thio(Ac CRAFQYHSKEQQC-NH₂), the thiol Cys side chain was protected with the trityl (Trt) group, which is removable with TFA (Andreu et al., 1994). The side chain of rest of trifunctional amino acids were protected with the common tBu-based groups, which are eliminated during the acidolysis with TFA, which also releases the peptide from the resin. TFA cleavage, work-up, and formation of the disulfide bridge between the free thiols, when applicable, was carried out as described elsewhere (An et al., 1998). N- and C-termini of all peptides were blocked with acetyl and amide blocking group, respectively, except for the four C-terminal Lys peptides (1)–(4). C-terminal Lys peptides were blocked only at their N-termini with the acetyl group. The free sulfonate group of cysteine (X) was blocked with acetamidomethyl blocking group (Acm, -CH₂NHCOCH₃).

NMR procedures

¹H-NMR spectra were collected at 500 MHz on a Bruker AM-500 spectrometer, as described (An et al., 1998). One-dimensional spectra were recorded at 298 K, with a spectral width of 7,246 Hz and 16K data points. Before processing the data, the FIDs were zero-filled to 32K, yielding a digital resolution of 0.2264 Hz. In ligand titration experiments, 256–1,024 scans were acquired to record spectra after each ligand addition. Suitable line-shaping was achieved via exponential or Gaussian function multiplication of the FID. The water signal was suppressed by presaturation between scans. Two-dimensional ¹H-NMR experiments were performed in the phase-sensitive mode using TPPI (Marion & Wüthrich, 1983). Two-dimensional chemical shift correlated (COSY) experiments (Aue et al., 1976; Wider et al., 1984) involved 512 × 4K data matrices, zero-filled to 4K along *t*₂, and multiplied by sine-bell functions along each dimension prior to Fourier transformation. As a control, COSY experiments were recorded at the beginning and at the end of each ligand titration experiment.

Determination of equilibrium association constant (*K_a*) values

Titration with NTP peptides were carried out at 298 K as outlined for similar experiments on CB-NTP in the accompanying paper

(An et al., 1998). Kringle samples were 0.7–1.0 mM in $^2\text{H}_2\text{O}$, pH* 7.2. Following each peptide addition, resolved kringle aromatic chemical shifts were monitored from 1D spectra. $[\text{K}]/[\text{peptide}]$ ratios were estimated from gravimetric determinations and corrected to “true” values by amino acid analysis after completion of each ligand titration experiment (An et al., 1998). K_a values were calculated as described (De Marco et al., 1987; An et al., 1998). Based on the ligand perturbation of kringle resonances and $[\text{ligand}]/[\text{kringle}]$ concentration ratios, changes in the chemical shifts were fitted to a Langmuir adsorption isotherm via a nonlinear regression routine based on the Levenberg–Marquardt algorithm, as described (An et al., 1998).

Computational modeling of the NTP-K4 complex

CB-NTP-K4 complex was generated using X-PLOR 3.1 (Brünger, 1988), starting from the ^1H -NMR solution structures of horse K4 (Cox et al., 1994) and CB-NTP (An et al., 1998). CB-NTP was manually docked to the K4 binding site, similarly as reported for a small ligand (Byeon et al., 1995), where the ϵ -amino group of the Lys50 was made to approach the Asp55 and Asp57 γCOO^- groups. The “best,” i.e., closest to the average, structures of NTP and K4 were used as the initial starting structures. Since NOEs between L-NTP and K4 were not observed, intermolecular distance constraints were not used in the calculation. Initially, the CB-NTP was manually placed in close proximity to the K4 binding site. Five hundred cycles of rigid-body energy minimization were applied to the complex, with bond distances and dihedral angles fixed for each molecule. Subsequently, the CB-NTP-K4 complex was subjected to a second round of 500 cycles of Powell energy minimization; at this stage, the residues in the K4 LBS, Asp55, Asp57, Trp62, Trp72, Lys36, Arg71, Tyr64, Phe74, and NTP residues within the stretch Glu35–Glu51 were given freedom to have their side chains move and interact. In order to account for the nonbonding interaction, Lennard–Jones and Coulombic electrostatic (with distance-dependent dielectric constant) potentials were used.

Acknowledgments

This work was supported by the U.S. Public Health Service, NIH grant HL-29409, the Spanish CICYT, grant PB95-1131, and the Swiss NSF, grants 31-39298 and 31-45816.98.

References

- An SSA, Marti D, Carreño C, Albericio F, Schaller J, Llinás M. 1998. Structural/functional properties of the Glu1–HSer57 N-terminal fragment of human plasminogen: Conformational characterization and interaction with kringle domains. *Protein Sci* 7:1947–1959.
- Andreu D, Albericio F, Solé NA, Munson MC, Ferrer M, Barany G. 1994. Peptide synthesis protocols. In: Pennington MW, Dunn BM, eds. *Methods in molecular biology*, Vol 35. Totowa, NJ: Humana Press. pp 91–169.
- Aue WP, Bartholdi E, Ernst RR. 1976. Two-dimensional spectroscopy: Application to nuclear magnetic resonance. *J Chem Phys* 64:2229–2246.
- Balciunas A, Fless GM, Scanu AM, Copeland RA. 1993. Interactions of a fluorescently labeled peptide with kringle domains in proteins. *J Protein Chem* 12:39–43.
- Belczewski AR, Laplaud PM, Chapman MJ, Koschinsky ML. 1996. The complete cDNA sequence encoding plasminogen from the European hedgehog (*Eriacus europaeus*). *Gene* 171:271–274.
- Berglund L, Andersen MD, Petersen TE. 1995. Cloning and characterization of the bovine plasminogen cDNA. *Int Dairy J* 5:593–603.
- Brünger AT. 1988. *X-PLOR version 3.3: A system for X-ray crystallography and NMR*. New Haven: Yale University Press.
- Byeon JIL, Kelley RF, Mulkerrin MG, An SSA, Llinás M. 1995. Ligand binding to the tissue-type plasminogen activator kringle 2 domain: Structural characterization by ^1H -NMR. *Biochemistry* 34:2739–2750.
- Cox M, Schaller J, Boelens R, Kaptein R, Rickli E, Llinás M. 1994. Kringle solution structures via NMR: Two-dimensional ^1H -NMR analysis of horse plasminogen kringle 4. *Chem Phys Lipids* 67/68:43–58.
- Degen SJ, Bell SM, Schaefer LA, Elliott RW. 1990. Characterization of the cDNA coding for mouse plasminogen and localization of the gene to mouse. *Genomics* 8:49–61.
- De Marco A, Petros AM, Laursen RA, Llinás M. 1987. Analysis of ligand-binding to the kringle 4 fragment from human plasminogen. *Eur Biophys J* 14:359–368.
- Forsgren M, Råden B, Israelsson M, Larsson K, Hedén L-O. 1987. Molecular cloning and characterization of a full-length cDNA clone for human plasminogen. *FEBS Lett* 213:254–260.
- Hochschwender SM, Laursen RA. 1981. The lysine binding sites of human plasminogen: Evidence for a critical tryptophan in the binding site of kringle 4. *J Biol Chem* 256:11172–11176.
- Horrevoets AJG, Smilde AE, Fredenburgh JC, Pannekoek H, Nesheim ME. 1995. The activation-resistant conformation of recombinant human plasminogen is stabilized by basic residues in the amino-terminal hinge region. *J Biol Chem* 270:15770–15776.
- Ichinose A. 1992. Multiple members of the plasminogen-apolipoprotein(a) gene family associated with thrombosis. *Biochemistry* 31:3113–3118.
- Lawn RM, Boonmark NW, Schwartz K, Lindahl GE, Wade DP, Byrne CD, Fong KJ, Meer K, Patthy L. 1995. The recurring evolution of lipoprotein(a): Insights from cloning of hedgehog apolipoprotein(a). *J Biol Chem* 270:24004–24009.
- Lerch PG, Rickli EE, Lergier W, Gillesen D. 1980. Localization of individual lysine binding regions in human plasminogen and investigation on their complex-forming properties. *Eur J Biochem* 107:7–13.
- Marion D, Wüthrich K. 1983. Application of phase sensitive two-dimensional correlated spectroscopy (COSY) for measurement of ^1H – ^1H spin–spin coupling constant in proteins. *Biochem Biophys Res Commun* 113:967–978.
- Markus G. 1996. Conformational changes in plasminogen, their effect on activation, and the agents that modulate activation rates: A review. *Fibrinolysis* 10:75–85.
- Marti D, Hu C-K, An SSA, von Haller P, Schaller J, Llinás M. 1997. Ligand preferences of kringle 2 and homologous domains of human plasminogen: Canvassing weak, intermediate and high affinity binding sites by ^1H -NMR. *Biochemistry* 36:11591–11604.
- Marti T, Schaller J, Rickli EE. 1985. Determination of the complete amino acid sequence of porcine miniplasminogen. *Eur J Biochem* 149:279–285.
- Rajan N. 1987. Interaction between human fibrin and plasminogen using synthetic peptides as models and the interaction between fibrin and tissue plasminogen activator [Doctoral Dissertation]. Boston University.
- Ramesh V, Rajan N, Laursen RA, Llinás M. 1995. Plasminogen kringle 4 binds the heptapeptide fragment 44–50 of the plasminogen N-terminal peptide. *Blood Coag Fibrinol* 6:207–218.
- Rejante MR, Byeon JIL, Llinás M. 1991. Ligand specificity of human plasminogen kringle 4. *Biochemistry* 30:11081–11092.
- Ryan TJ, Keegan MC. 1985. Photoaffinity labeling of functionally different lysine-binding sites in human plasminogen and plasmin. *Biochim Biophys Acta* 830:187–194.
- Schaller J, Marti T, Rösset SJ, Kämpfer U, Rickli EE. 1987. Amino acid sequence of the heavy chain of porcine plasmin. Comparison of the carbohydrate attachment sites with the human and bovine species. *Fibrinolysis* 1:91–102.
- Schaller J, Moser PW, Dannegger-Müller GAK, Rösset SJ, Kämpfer U, Rickli EE. 1985. Complete amino acid sequence of bovine plasminogen: Comparison with human plasminogen. *Eur J Biochem* 149:267–278.
- Sjöholm I, Wiman B, Wallén P. 1973. Studies on the conformational changes of plasminogen induced during activation to plasmin and by 6-aminohexanoic acid. *Eur J Biochem* 30:471–479.
- Söndel S, Hu CK, Marti D, Affolter M, Schaller J, Llinás M, Rickli EE. 1996. Recombinant gene expression and ^1H NMR characteristics of the kringle (2 + 3) supermodule: Spectroscopic/functional individuality of plasminogen kringle domains. *Biochemistry* 35:2357–2364.
- Takada Y, Urano T, Takada A. 1993. Conformational change of plasminogen: Effects of N-terminal peptides of Glu-plasminogen. *Thrombosis Res* 70:151–159.
- Tomlinson JE, McLean JW, Lawn RM. 1989. Rhesus monkey apolipoprotein(a): Sequence, evolution, and sites of synthesis. *J Biol Chem* 264:5957–5965.
- Urano T, Takada Y, Takada A. 1991. Effects of N-terminal peptide of Glu-plasminogen on the activation of Glu-plasminogen and its conversion to Lys-plasminogen. *Thrombosis Res* 61:349–359.

- Wallén P. 1980. Biochemistry of plasminogen. In: Kline DL, Reddy KNN, eds. *Fibrinolysis*. Boca Ratón, Florida: CRC Press. pp 1–24.
- Weissbach L, Treadwell BV. 1992. A plasminogen-related gene is expressed in cancer cells. *Biochem Biophys Res Commun* 186:1108–1114.
- Wider G, Macura S, Kumar A, Ernst EE, Wüthrich K. 1984. Homonuclear two-dimensional ^1H NMR of proteins: Experimental procedures. *J Mag Reson* 56:207–234.
- Wiman B, Almqvist Å, Rånby M. 1989. The non-covalent interaction between plasmin and α_2 -antiplasmin. *Fibrinolysis* 3:231–235.
- Wiman B, Collen D. 1978. Molecular mechanism of physiological fibrinolysis. *Nature* 272:549–550.
- Wiman B, Wallén P. 1975. Structural relationships between “Glutamic Acid” and “Lysine” forms of human plasminogen and their interaction with the NH_2 -terminal activation peptide as studied by affinity chromatography. *Eur J Biochem* 50:489–494.