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Lysine/Fibrin Binding Sites of Kringles Modeled After the Structure of Kringle 1 of Prothrombin

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ABSTRACT **The Lys binding site of kringle 1 and 4 (K1 and K4) of plasminogen (PG) has been modeled on the basis of the three-dimensional structure of kringle 1 of prothrombin and 300- and 600-MHz proton nuclear magnetic resonance observations. These structures were then compared to the corresponding regions of modeled kringle 1 and 2 of tissue plasminogen activator (PA). The coordinates of the modeled structures have been refined by energy minimization in the presence and absence of ϵ-aminocaproic acid ligand in order basically to remove unacceptable van der Waals contacts. The binding site is characterized by an apparent dipolar surface, the polar parts of which are separated by a hydrophobic region of highly conserved aromatic residues. Zwitterionic ligands such as Lys and ϵ-aminocaproic acid form ion pair interactions with Asp55 and Asp57 located on the dipolar surface; the latter are also conserved in all the Lys binding kringles. The cationic center of the dipolar surface is Arg71, in the case of PGK4, and is composed of Arg34 and Arg71 in PGK1. The doubly charged anionic/cationic interaction centers of the latter might account for the larger binding constants of PGK1 for like-ligands but the modeling suggests that PGK4 might be kinetically faster in binding bulkier ligands. The binding site region of PAK2, which also binds Lys, resembles those of PGK1 and PGK4. Since PAK2 lacks both cationic center Arg residues, ligand carboxylate binding appears to be accomplished though an imidazolium ion of His64, which is located just below the outer surface of the kringle.**

Key words: **molecular modeling, energy minimization, lysine/fibrin binding, kringle structures, plasminogen, tissue plasminogen activator**

INTRODUCTION

Kringle structures are highly conserved, three-disulfide, triple-loop polypeptides (Fig. 1) that occur 11 times in five different proteins of blood coagulation and fibrinolysis. They are found singly in urokinase[1,2] and factor XII,[3] as pairs in prothrombin (PT)[4] and tissue-type plasminogen activator (PA)[5] and multiply, a spectacular five times in plasminogen (PG)[6] (see also Note Added in Proof). In addition, a kringle-like structure also occurs in haptoglobin, a plasma glycoprotein composed of two chains.[7] Kringles and other structural domains (epidermal growth factor, Ca^{2+}-binding, finger-type)[3,8] often accompany catalytic segments as large noncatalytic amino terminal regions, and they appear to display recognition and generally impart specificity to certain biological processes by interacting with other macromolecules. These noncatalytic regions are thought to have evolved from a series of smaller tandem duplications or from the individual fusion of separately evolved components.[8] Thus, the second kringle (K2) of PT substrate of the prothrombinase complex binds to the heavy chain of membrane-bound factor Va[9,10], and K1 and K4 of PG[11–13] and K2 of PA[14–16] bind to fibrin, lysine, or ω-amino-carboxylic acids in general, particularly ϵ-aminocaproic acid (ϵACA). Since ϵACA is a close analog to lysine, which is involved in the kringle binding of fibrin, it has been chosen as the substrate in the modeling studies described below. In contrast to the foregoing, K5 of PG binds benzamidine[17] but not lysine nor arginine (T. Thewes, M. Llinás, unpublished observations).

Residues Asp57 and Arg71 of PGK4 (Table I) have been implicated as the ionic centers that interact with zwitterions like Lys and ϵACA in the primary binding process.[13] Since we have recently solved the 2.8 Å resolution three-dimensional structure of PT fragment 1 (F1)[18] which contains K1 of PT, the fibrin/Lys binding properties of kringles led us in a natural way to investigate the binding site region of kringles in terms of the folding and structure of the kringle of F1 (PTK1) utilizing computer graphics modeling modulated to conform with 300- and 600-MHz ^{1}H-nuclear magnetic resonance (NMR) observations.[19–23] The resulting structures were then submitted to a fairly restrained and mild weighted energy refinement basically to optimize van der Waals contacts and electrostatic interactions of the binding site, with and without ligand, and to estimate the relative contributions of energy components.

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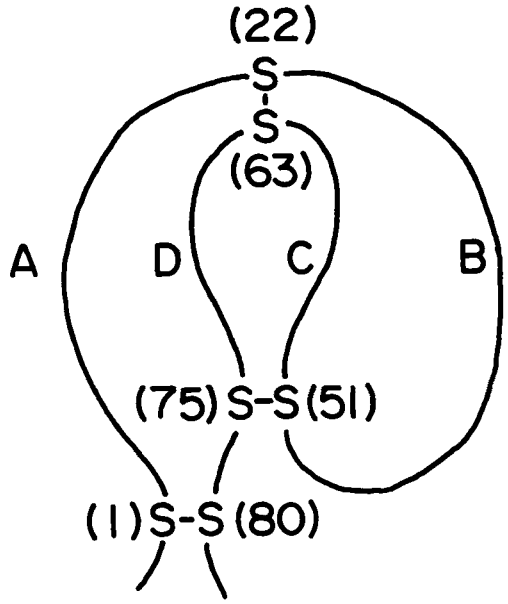


Fig. 1. The three-disulfide triple-loop kringle structure. Loop segments designated A, B, C, D.

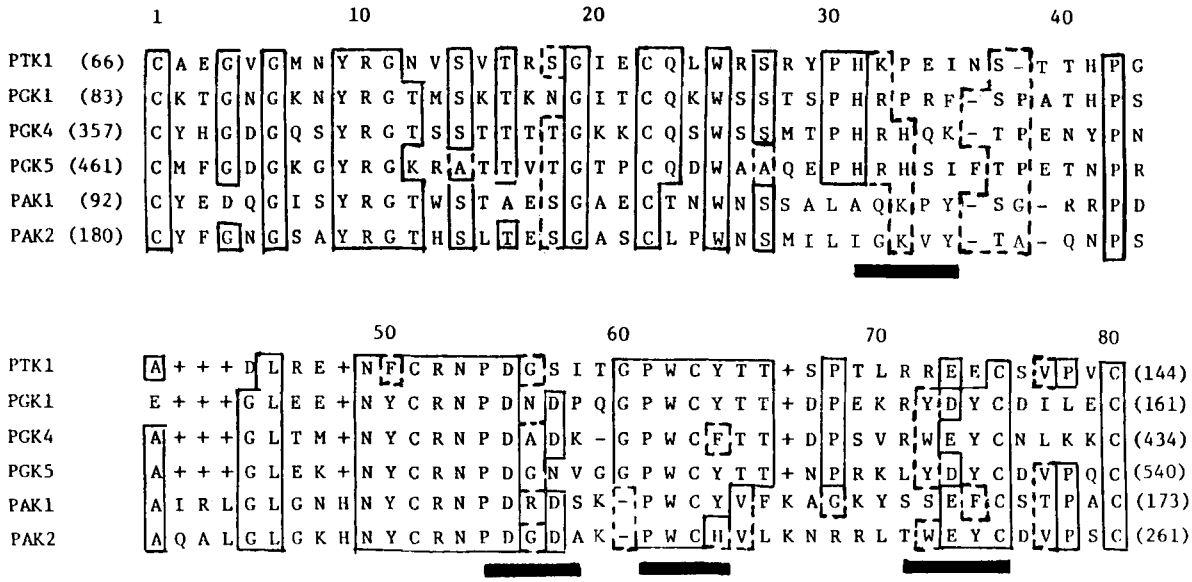
is very similar to kringles that bind to fibrin/Lys.[24,25] Therefore, the three-dimensional structures of various kringle sequences were approximated from the PTK1 structure[18] using molecular modeling methods with an Evans & Sutherland PS350 interactive computer graphics display driven by FRODO.[26] First, the PTK1 sequence in the Lys binding region (the vicinity of crucial Asp57 and Arg71 of K4 of PG, Table I) was changed to correspond to that of the kringle in question. In this maneuver, side chain positions of like atoms between PTK1 and the new kringle were kept constant and served as guide coordinates for replaced side chains. Thus, $\chi 1$ of Ser57 of PTK1[18] was assigned to $\chi 1$ of Asp57 of PGK4.* In the absence of guide atoms, atoms of new side chains were placed in extended or conformationally idealized positions. Although such procedures were reasonable and suggestive for many of the side chains, nonetheless, they were not entirely satisfactory as some unacceptably close van der Waals contacts were also generated. Throughout these initial manipulations of amino acid sequence changes, the main chain folding of the PTK1 was kept unaltered.

PROCEDURES

Although lacking measurable fibrin/Lys binding capability, PTK1 is likely to exhibit a conformation that

*This orientation for Asp57 later also proved to be suitable for ligand interaction.

TABLE I. Primary Structures of Homologous Kringles From Bovine Prothrombin, Human Plasminogen, and Tissue Plasminogen Activator[a]



[a]The numbering of residues (1–80) is standardized to the plasminogen PGK5 sequence in accordance with convention followed previously. Sites inserted or deleted relative to the K5 sequence are indicated with + and −, respectively. Inserted residues are numbered according to the preceding, non-inserted residue number, followed by a, b, . . ., etc. Thus, PAK1 has Ala44, Ile44a, Arg44b, and Leu44c, as well as Asn48 and His48a, and Phe66 and Lys66a. Conserved amino acids are boxed, homologous conservation is dotted. Segments involved in the fibrin/Lys binding region are underlined with heavy trace. Numbers in parentheses indicate numbering of Cys1 and Cys80 within primary sequence of parent protein.

The kringle structures thus formed were also modulated in the case of K1 and K4 of PG to be made consistent with 300- and 600-MHz proton NMR results of the kringles with and without ligands bound at the Lys binding site. Aromatic proton NMR spectra of these two kringles have been fully analyzed, and resonances have been assigned to specific residues.[24,25,27,28] Presence of aliphatic or aromatic ligands induce large shifts in the ring resonances of several aromatic residues. In PGK1, Phe36, Trp62 and Tyr72 while in PGK4 Trp62, Phe64 and Trp72 are the most perturbed by ligand presence. Reciprocally, the aromatic side chains at the binding site consistently shift methylene proton resonances of bound, aliphatic ligands.[20–22] Protein-ligand saturation transfer experiments confirmed the direct involvement of those residues and assisted further in adjusting the initially modeled structures. Most of the proton Overhauser constraints could be easily satisfied simply by facile torsional rotations of side chains. For instance, the phenolic ring of Tyr74 of PGK1 can readily be positioned approximately perpendicular to and within 4.6 Å of the methine H2 proton of the indole of Trp62.[23] Similarly, the aromatic ring of Phe36 can be rotated about its $\chi 1$ and $\chi 2$ angles to impinge upon the fibrin/Lys binding site.[23]

The next step in the modeling was to dock a ligand ϵACA molecule between the anionic carboxylate Asp57 and cationic guanidinium Arg71 centers of the more refined models. This proved to be relatively straightforward since these kringle charge centers are about 12.5 Å apart suggesting that the Lys-type ligands are in an extended conformation in the protein-ligand complex. The observation is also consistent with the binding constants of various ligands of differing overall length: as the length between charges deviates from an optimum of about 6.8 Å,† the ligand binding constants decrease.[29] Minor torsional adjustments of Asp57 and Arg71 and several other side chains in the ligand-docked binding site then led to satisfactory ion pair and van der Waals interactions between ligand and protein. In the case of PGK4, an additional rotation of about 160° around $\chi 2$ of Trp72 was applied to change the guide orientation so as to remove the six-membered ring of the indole from collision with ligand; this also brought the indole nitrogen to the surface and directed the nonpolar six-membered ring toward other aromatic residues in the interior. Again, throughout this second level of adjustments, the integrity of the main chain folding of PTK1 was preserved.

The final step to the modeling was to refine the energy of the structures, with and without ϵACA ligand, using the Adopted Basis Newton-Raphson energy minimization procedure in CHARMM.[30] The residues in the kringle were divided into three groups. The first group consisted of replaced residues at the binding site (Table I). Of the remaining residues, which were those of PTK1, those residues with side chain atoms within 5 Å of any ligand or group I atom formed the second group,‡ and those farther away formed the third group. During energy minimization, ligand atoms were allowed to be totally free, while kringle atoms were constrained. Group I atoms of the polypeptide backbone were constrained with an isotropic harmonic potential of 20.0 kcal/mol/Å for the first 200 steps of minimization and then with 10.0 and 5.0 kcal/mol/Å for the second and third sets of 200 steps. Side chain atoms of this group were given more freedom and were only constrained with 10.0, 5.0, and 2.0 kcal/mol/Å, respectively, in the three refinement stages. All atoms in group II were constrained with isotropic harmonic potential of 20.0 (backbone) and 10.0 (side chain), while those of group III were held at fixed crystallographic positions. An 8.0-Å cut-off was used for the electrostatic and van der Waals terms. The total energy of the modeled systems decreased asymptotically, and in the last stages of the refinement, they decreased by less than 0.01 kcal/mol per refinement step.

RESULTS AND DISCUSSION

A schematic of the three-disulfide, triple-loop kringle structure is shown in Figure 1 where the different segments of the loop structures have been designated with letters. To simplify the comparative analysis of the various homologs, we adhere to an absolute numbering of residues based on the PGK5 sequence (Table I). The kringle structures studied here were K1 and K4 of PG and both kringles of PA; K4 has a deletion at site 59 with respect to PTK1 in the C segment of the inner loop, while all the loops of K1 of PT and K1 of PG possess the same number of residues. Both kringles of PA differ from PTK1 by having three additional residues in the B segment of the outer kringle loop (sites 44a,b,c) and an insertion (site 48a)/deletion (site 60) in the B and C segments, respectively. Only the deletion in the C segment at site 60 presently appears to have sound basis by virtue of crowding in a peptide turn.[18]

The Lys binding region can be defined as bounded by residues 31–35, 54–58, 61–64, and 71–75 (Table I), and it is located on the surface of one of the oblate faces of the kringle (which approximates an oblate ellipsoid 11 × 30 × 30A).[18] Thus, the binding site region essentially corresponds to the inner loop of the kringle sequence[13,19,20] (C and D, Fig. 1). More specifically, the site is below the three-dimensional loop formed by the C segment of the inner kringle loop

†Calculated from nitrogen atom to carbon of carboxylate group.

‡There are 32 residues in this group with about half differing in sequence between PT and PG kringles.

(Fig. 1) and runs parallel to the antiparallel β-strand loop formed by the D segment.[18]

Kringle 4 of PG

A stereoview of the modeled fibrin/Lys binding region of the PGK4 is shown in Figure 2a. Most of the differences from the PTK1 sequence occur around the periphery of the binding site (Table I), with four aromatic residues forming the center of the site (Trp62, Phe64, Trp72, Tyr74) (Fig. 2a). In addition, Trp25, in the next layer, is in contact with Trp62. Such aromatic clustering is fairly common[31,32] in proteins. An ϵACA molecule has also been docked in the site between the Asp57 anionic and Arg71 cationic centers. In order to produce ion pair interactions with these residues in the liganded kringle, an extended conformation had to be used for ϵACA. Since residue 57 is a Ser in PTK1, which afforded excellent guide coordinates for ligand interaction of Asp57,* and since Arg71 is conserved, only minor torsional rotations

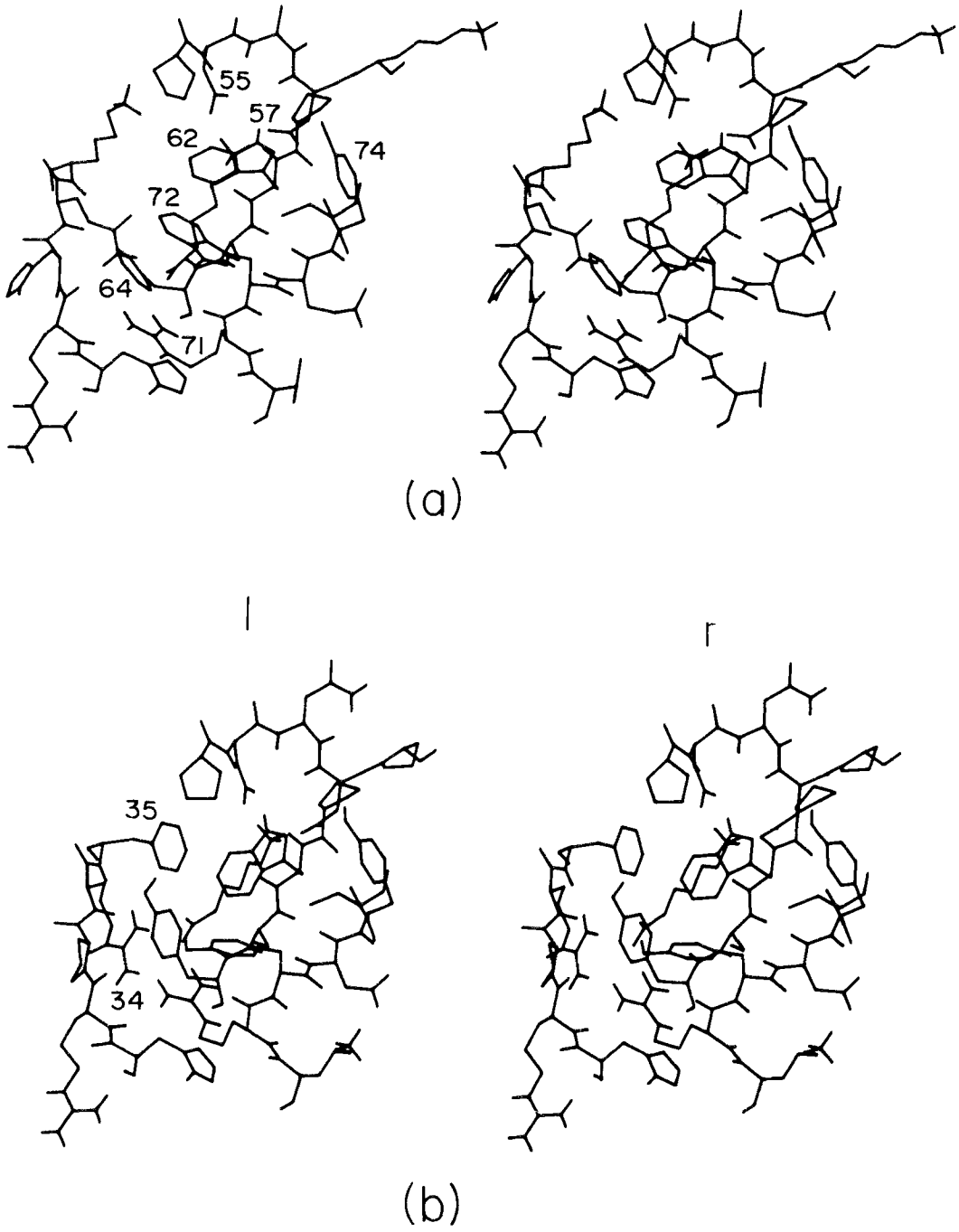


Fig. 2. Stereoviews of modeled-energy-minimized Lys binding sites. **a:** PGK4; and **b:** PGK1, each occupied by ϵACA.

were required in reorienting the two side chains to improve the ion pair interactions with ligand. Although it is difficult to predict the detailed effect of the π electron distributions of the aromatic residues in the central stack lining the binding site, to a first approximation they appear to provide a highly nonpolar environment for the five methylene groups of ϵACA, particularly the indole of Trp72, which is spanned by the ligand (Fig. 2a). Last and very interestingly, the Arg32-Pro33-Arg34 sequence of PGK1, which lies adjacent to the Lys binding site (Fig. 2a and b), has been implicated by fibrin- and Lys-Sepharose affinity binding experiments[33] to be involved in fibrin binding. As the exact nature of the specificity marker of fibrin becomes clear, the modeling can be extended to include the Arg32-Pro33-Arg34 sequence. The marker appears to be more complex than simply a Lys residue, but Lys appears essential. A heptapeptide, Ala-Phe-Gln-Tyr-His-Ser-Lys, has been shown to bind to plasminogen,[34] PGK1,[11] and PGK4,** while the undecapeptide Aα-149-159 fragment of fibrinogen, with its striking alternating polar/nonpolar sequence, Arg-Leu-Glu-Val-Asp-Ile-Asp-Ile-Lys-Ile-Arg, has been shown to accelerate tissue plasminogen activator activation of plasminogen similarly to fibrin.[35] Either or both of these polypeptides might harbor the fibrin specificity marker.

Kringle 1 of PG

The proton Overhauser constraints[23] of PGK1 indicate that the ligand interacts directly with Phe35,¶ Trp62, and Tyr72. Once again, the folding of PTK1 was kept intact in modeling, and minor torsional adjustments of Trp62 and Tyr72 could produce good contacts with ligand. However, the Phe35 residue, which replaces Ile35 in PTK1 and which corresponds to a dominant NMR ligand interaction, required quite substantial torsional rotations to orient the phenyl ring into the vicinity of the ligand. Even so, the ligand contacts could be improved further by reorienting and translating the ligand slightly with respect to its modeled position in PGK4 (compare Figs. 2a and b). As with PGK4, most of the sequence differences of PGK1 relative to that of PTK1 occur around the periphery of the site with the Phe35 replacement increasing the number of central aromatic residues of the site to five. A stereoview of the modeled binding region of PGK1 is shown in Figure 2b.

In the placement of the ligand and Phe35 to satisfy the Overhauser constraint, the carboxyl group of ϵACA still proves to lie within ion pair interaction distance of Arg71; however, in addition, it is now also close to the guanidinium group of Arg34, especially with a few minor torsional readjustments of the side chain of the latter (Fig. 2b). Thus, it appears that the cationic charge center of PGK1 differs from that of PGK4 in being made up of two charged guanidinium groups that can impinge upon the carboxylate of ϵACA. Moreover, the anionic center apparently mirrors the cationic one in being composed of two anionic carboxylate groups of neighboring Asp55 and Asp57 residues. In fact, the latter residues are conserved in the fibrin/Lys binding kringles so that the doubly charged anionic center might be of a general nature and also operative in PGK4 (see "Effect of Energy Refinement on the Modeled Structures"). A role for Asp55 in ligand binding had previously been postulated on the basis of homology criteria.[36]

That Arg34 also participates with Arg71 in making up the cationic center of the fibrin/Lys binding site of PGK1 is consistent with the fact that modification of Arg34 with 1,2 cyclohexanediol abolishes both fibrin and Lys binding, whereas similar modification of Arg32 leads only to loss of fibrin binding without affecting Lys binding.[33] Position 34 is either not involved in Lys binding in PGK4 or at least is not an important factor. This is because in chicken PGK4 this position is occupied by Asn, which is additionally glycosylated, but yet the kringle displays Lys binding properties.[37]

Comparison of K4 and K1

Space-filling representations of the fibrin/Lys binding sites of PGK4 and PGK1 are shown in Figures 3 and 4, respectively. The most conspicuous aspect of these drawings, beside the hydrophobic methylene recess (Figs. 3b, 4b) on the surface occupied with ϵACA ligand (Figs. 3a, 4a) and lined with aromatic residues (Figs. 3a, 4a), is the segregation and apparent immiscibility of oxygen and nitrogen atoms surrounding the binding site on the surface. The carboxylate end of the ligand is tucked into the edge of the highly electropositive portion of this dipolar surface near the guanidinium groups. In contrast, the amino terminal of ϵACA is pincered in by the negative carboxylate groups, while the methylene groups span a relatively neutral zone separating the charged polar portions, thus intimately interacting with the exposed aromatic side chains (Figs. 3, 4). The dipolar surface could be of functional significance in recognizing at long range the marker of the fibrin ligand site and in aiding in the approximate orientation and alignment of the kringle. This would lead to a better-defined interaction upon close approach to fibrin and could also be a major contributing factor to the resultant stability of interaction of the docked fibrin-kringle complex. From the obvious complexity of the atomic distribution in the dipolar surface, it is clear that rational alterations of the surface using bioengineering methods will have to be equally sophisticated.

**V. Ramesh, R.A. Laursen, M. Llinás, unpublished observations.

¶In an earlier publication,[23] Phe35 was labelled Phe36. See footnote ¶¶.

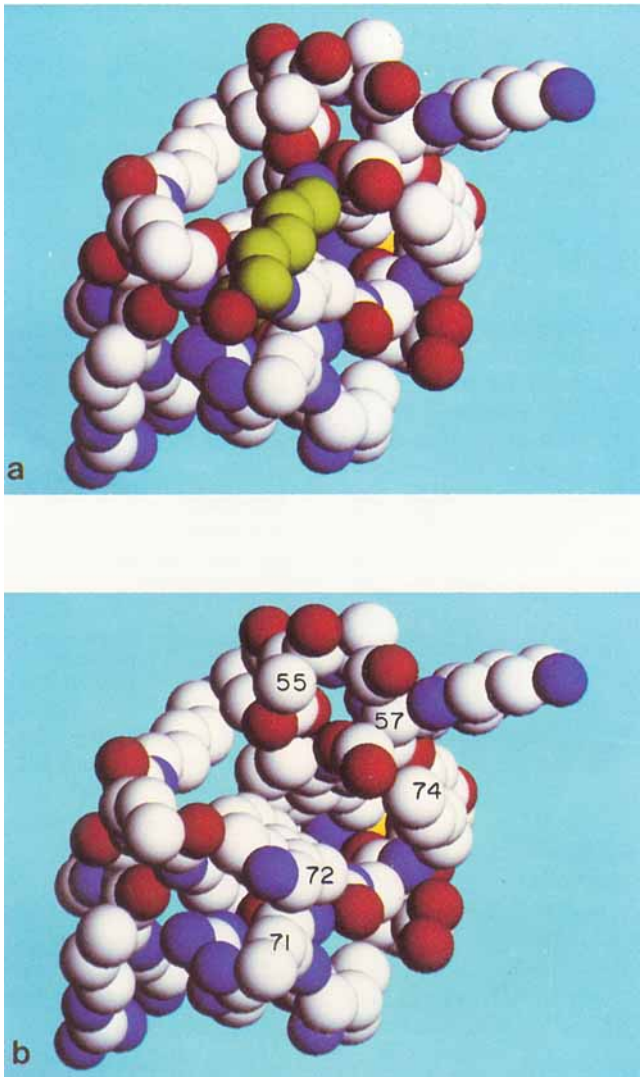


Fig. 3. Computer-generated space-filling views of modeled-energy-minimized Lys binding site of PGK4. **a:** Occupied with εACA (green); **b:** unoccupied by ligand; carbon-gray, nitrogen-blue, oxygen-red, sulfur-yellow; selected residues common to both panels numbered.

From Figures 3a and 4a it is apparent that the Lys binding site region of PGK4, with the absence of Phe at position 35, is considerably more open and not nearly as restricted as the methylene recess exposed by PGK1. The restriction in the latter is basically a consequence of the presence of Phe35 and is probably one reason why PGK1 displays a better binding constant for p-benzylamine sulfonic acid.[38] Another reason for better binding might be the enhanced polarity of the cationic region of the binding site of PGK1 where it is composed of Arg34 and Arg71 compared to only the latter in PGK4. Conversely, however, PGK4 might be kinetically faster and may display larger values for the k_{on} parameters[§] of binding, especially for bulkier ligands such as 4-aminomethyl(cyclohexane)carboxylic acid.[22]

PAK1 and PAK2

Both PA kringles possess the necessary Asp55 and Asp57 of the fibrin/Lys binding site but neither has an Arg at positions 34 and 71 of the cationic center (Table I). Instead, PAK1 fills these sites with Pro34 and Ser71 (Fig. 5a), while PAK2 carries Val and Thr at these positions, respectively (Fig. 5b). Nonetheless, the latter displays fibrin/Lys binding.[14–16] Although it appears that Lys33 in the PA kringles might play a similar role to Arg34 of PGK1 in fibrin/Lys binding,[23,33] the situation is complicated by the substitutions at 71, which are certain to diminish the effectiveness of the binding in the cationic site, and the deletion at 36 in the region (Table I).[¶¶] An examination of the modeled structure of the two PA kringles shows that in the case of PAK2, the cationic

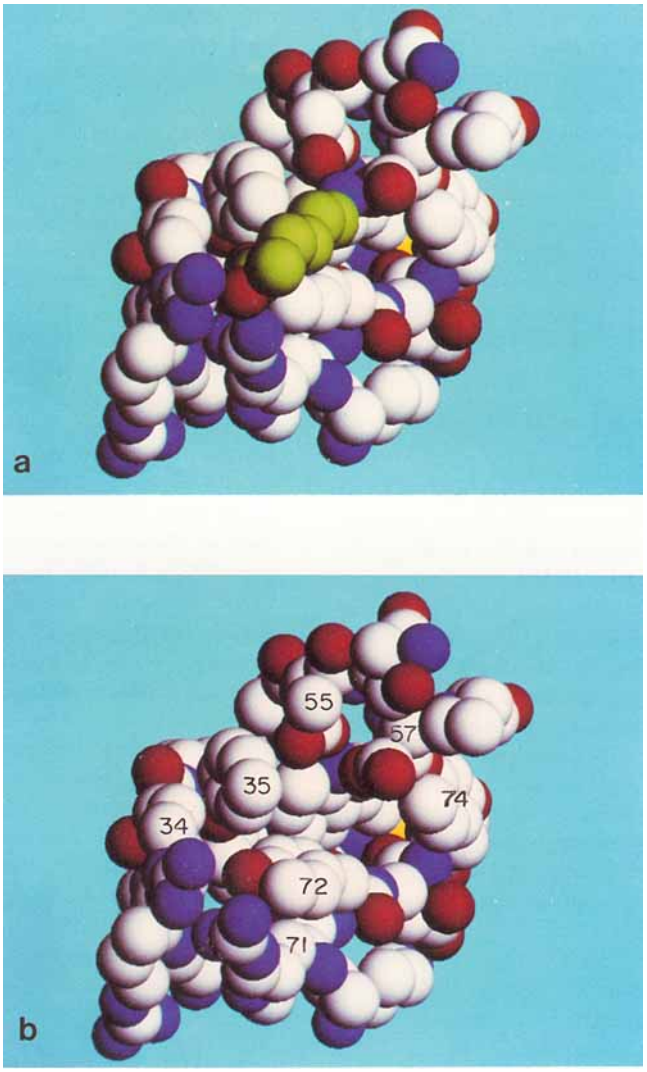


Fig. 4. Computer-generated space-filling views of modeled-energy-minimized Lys binding site of PGK1. **a:** Occupied by ϵACA (green); **b:** unoccupied by ligand; colors as in Figure 3; selected residues common to both panels numbered.

center might be provided by an imidazolium ion from His64 (Table I). This residue substitutes for Tyr64 and Phe64 in PGK1 and PGK4, respectively, and is thus located just below the surface of the binding site periphery adjacent to the Arg71 position (Fig. 5b). Therefore, it might easily provide a positively charged center from within to pair with a negative carboxylate group of the ligand. Although it would seem that Lys33 might be involved in binding since it is conveniently located in the vicinity, it appears not to. This is because its Cα-Cβ link is directed away from the binding site, so that an unlikely conformation would be required for the side chain. This is consistent with the lack of fibrin/Lys binding of PAK1 (as evidenced through lysine-Sepharose),[16] which also has Lys33.

Another position that might be of consequence in ligand binding is that of 72, which contains an aromatic residue in all the fibrin/Lys binding kringles (Table I). This all-important residue appears to provide an aromatic or hydrophobic platform, which the methylene groups of the ligand span (Figs 3b,4b and in guide orientation in 5b) and which is enclosed from two sides by other aromatic residues (35, 62, 64, and 74), creating an elongated aromatic recess on the surface. Indeed, chemical modification of Trp72 in PGK4 leads to loss of Lys-binding capability,[12] and

benzamidine binding to PGK5 consistently involves Tyr72.[39] The presence of an aromatic residue at 35 in this region does not appear to be crucial for binding since both PGK1 and PGK4 bind fibrin/Lys (Table I). However, the same might not apply for position 72, which contains a Ser residue in PAK1 (Fig. 5a), thus compounding the problem of the absence of Arg at 71 or an electropositive environment in the region. The combination might well be the reason for the absence of fibrin/Lys binding in this kringle. This is also evident from a comparison of Figures 3a, 4a, and 5a, which show that PAK1 is strikingly different from PGK1 and PGK4 in the binding site region.

Effect of Energy Refinement on the Modeled Structures

The energy minimizations carried out on the modeled structures are significant on a relative, but not absolute, basis; only the ligand and the binding site region were allowed to vary, and the remaining part of the structure was taken to be constant and that of PTK1. Thus, the energies are only indicative of the fibrin/Lys binding site region. Moreover, contributions of solvent were not included because 1) the PTK1 structure was solvent-flattened[18] and the relatively low resolution (2.8 Å) did not warrant refinement and location of solvent, and 2) even if the solvent structure of PTK1 were known, it would most likely be fairly different in the other kringles. Ignoring the solvent can be expected to produce a large effect on the magnitudes of electrostatic interactions. However, their relative values should prove suggestive when comparing PGK1 and PGK4.

Since the trial structures for energy refinement were basically raw computer graphics creations with only manual intercession to impose structural NMR constrains,[23] newly created van der Waals contacts were not particularly optimal; this was reflected by initial positive total energies. However, the van der Waals and electrostatic contributions decreased rapidly, with minimization producing negative total energy and generally acceptable van der Waals contacts. Decreasing the weighting scheme in the second round of minimization enhanced the decrease of energies even further.

The final energies of the binding sites and their components are listed in Table II from which it is apparent that the principal difference between the liganded and unliganded state is due to the electrostatic contribution corresponding to the zwitterionic interaction of ligand with the cationic and anionic centers of the fibrin/Lys binding site. This difference is of course expected to be much smaller in magnitude if solvent molecules are included in calculations, since the electrostatic component of the energy in the unliganded state would be more negative. Moreover, the solvation effect will be greater for PGK1 compared to PGK4 because position 34 of PGK1 is a charged Arg, whereas it is a polar but uncharged Gln in PGK4, thus reducing the difference. It can also be seen from Table II that the calculations imply that contributions from van der Waals interactions to ligand binding are practically nonexistent. This is consistent with the fact that only Cα, and possibly Cβ, of ϵACA make a van der Waals contact with the aromatic residue in position 72 (Figs. 3a, 4a). From Table II, it can also be seen that the energies of the binding site region of the PA kringles calculate favorably compared to unliganded PGK1 and PGK4 even though the modeled PA structures were just computer graphics simulations and not modulated to conform to NMR or other information. The rms differences between modeled and minimized coordinates are given in Table III for various kringles and are displayed graphically in Figure 6 for PGK4 in the presence of ϵACA. The apparent large shifts of the ϵACA (Table III) are due to a fairly large rotation ($\sim 90°$) of the ligand about the axis of the extended conformation,[§§] along with a translational component in the case of PGK4 (Fig. 6). With the latter, the ligand was originally simply docked between Asp57 and Arg71, but ϵACA moved such as to place the amino group of ϵACA equidistant between Asp55 and Asp57 and the carboxyl group to within hydrogen bonding distance of Gln34, while maintaining an ion pair relationship with Arg71. Thus, it appears that two Asp residues are also operative in the anionic site of PGK4 in a fashion similar to that of PGK1. Another significant and reassuring result of the calculations was that the constraint-free ϵACA remained bound to the PG kringles. Moreover, only minor structural or conformational changes appear to accompany ligand binding (rms $\Delta \sim 0.06$ and 0.2 Å for main and side chains, respectively) consistent with NMR observations.[19-23] Therefore, the binding site must exist essentially preformed in the native kringle. From the difference in solvent-free energies of liganded and native PGK1 and PGK4 and those of ϵACA in Table II, it can be seen that the relative binding energy of PGK1 is about 30% greater than that of PGK4. This difference in the binding energies will be somewhat reduced when the differential solvation effects mentioned above are included

§§This was also suggested by space-filling views of the pre-energy refined strcture.

§ligand + kringle $\rightleftarrows$ ligand–kringle; $k_{on} = K\, k_{off}$ where K is the equilibrium binding constant.

¶¶In fact, it appears that the deletion at 36 (Table I) might simply be an artifact of choosing PGK5 as the basis of the numbering scheme; an insertion at 34 or 35 of PGK5 would serve to align Phe36 of PGK5 with the aromatic residues of position 35. The nagging question of deletions/insertions will only be completely answered when the folded structures of a number of different kringles are compared and aligned.

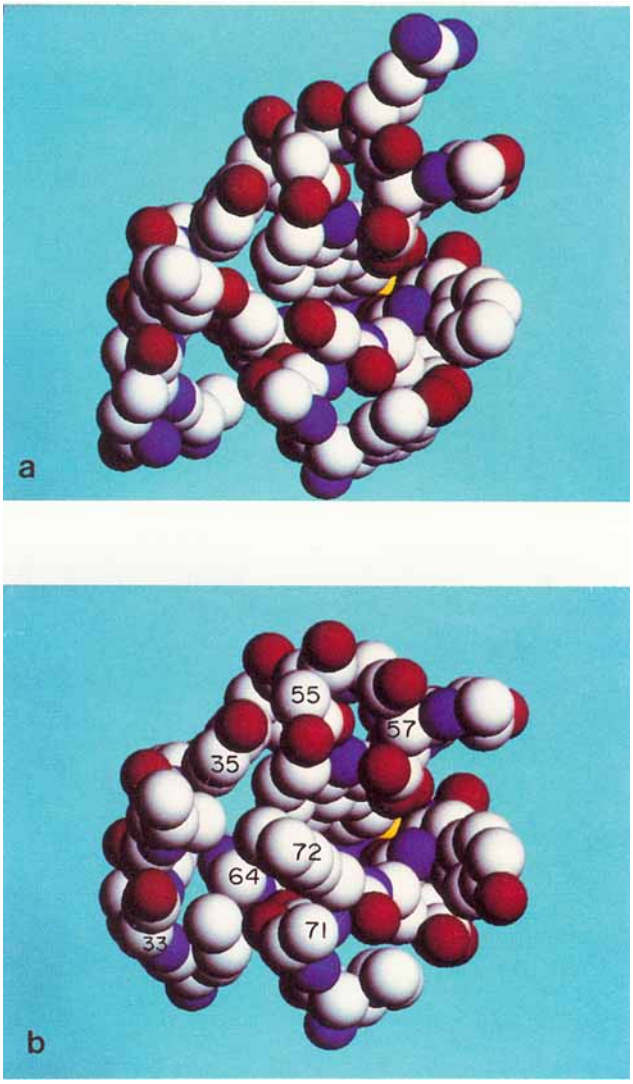


Fig. 5. Computer-generated space-filling views of modeled-energy-minimized Lys binding sites of PAK1 (**a**) and PAK2 (**b**). Colors as in Figure 3; selected residues common to both panels numbered.

in the calculations, and it will be considerably small in magnitude with solvation so that it should then correspond closely to the observed difference in the binding of ϵACA to PGK1 and PGK4 for like-ligands.[29,38]

Last, the changes due to energy minimization of the group I residues of the PA kringles are significantly smaller than those of PG (Table III) because the side chains were not manipulated to conform to NMR observations prior to refinement. The difference between the PG and PA kringles is due almost entirely to side chains (Table III, footnotes * and ‡). Also of note is the fact that when group II residues of PTK1 were replaced with the sequence of PGK1, the rms shifts during energy refinement for this group were half of those in Table III for the main chain (~0.2 Å) and even less for side chains (~0.25 Å). However, the remainder of the binding site (group I) and the ligand stayed essentially the same.

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TABLE II. Energy Components (kcal/Mol) of the Lys/Fibrin Binding Site*

Structure	E_t	E_B	E_T	E_P	E_I	E_{NB}	E_{EL}	E_{HB}
(PGK1: ϵACA)	−539	26	143	138	41	−323	−507	−56
PGK1	−376	24	139	136	40	−321	−341	−53
(PGK4: ϵACA)	−482	25	151	130	42	−333	−447	−51
PGK4	−360	24	149	129	40	−329	−323	−49
PAK1	−403	22	127	117	36	−319	−333	−53
PAK2	−372	22	126	114	37	−315	−302	−54
ϵACA	−4	~0	~0	~0	~0	−2	−2	~0
ΔEK1†	−159	1	4	2	1	0	−164	−3
ΔEK4†	−118	1	2	1	2	−2	−122	−2

*E_t = total energy; E_b = bond; E_T = angles; E_P = torsions; E_I = improper torsions (planarity, chirality); E_{NB} = van der Waals; E_{EL} = electrostatic; E_{HB} = hydrogen bond.
†$\Delta E_{kj} = (K_j$: ϵACA) − (K_j + ϵACA), j = 1 and 4.

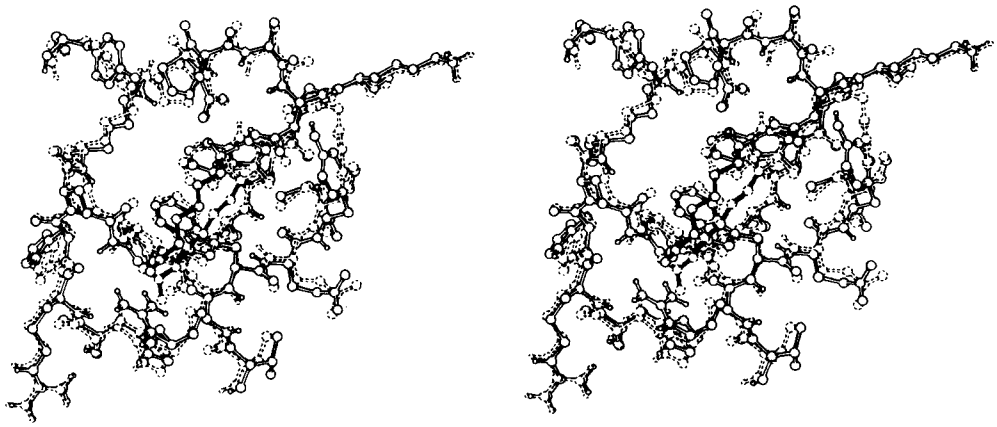

Fig. 6. Stereoview of modeled Lys binding site of PGK4 before (dotted) and after (solid) energy minimization. ϵACA ligand shown with solid bonds; hydrogen atom positions generated in structure prior to refinement.

TABLE III. rms Changes Caused by Energy Minimization

Structure	Ligand(A)	Group I (A)*	Group II (A)†
PGK1 + ϵACA	1.79	0.75	0.41
PGK1	—	0.74	0.41
PGK4 + ϵACA	2.40	0.80	0.40
PGK4	—	0.80	0.40
PAK1	—	0.57‡	0.37
PAK2	—	0.57‡	0.36

*Main chain ~ 0.35 Å, side chain ~ 0.85 Å.
†Main chain ~ 0.25 Å, side chain ~ 0.45 Å.
‡Main chain ~ 0.35 Å, side chain ~ 0.65 Å.

NOTE ADDED IN PROOF

Recent sequencing of apolipoprotein (a) shows remarkably that it essentially contains 37 copies of PGK4 and one of PGK5 (McLean, J.W., Tomlinson, J.E., Wun-Jing, K., Eaton, D.L., Chen, E.Y., Fless, G.M., Scanu, A.M., Lawn, R.M. c-DNA sequence of human apolipoprotein (a) is homologous to plasminogen. Nature 330:132–137, 1987.

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