Iterative Optimization of High-Affinity Protease Inhibitors Using Phage Display. 2. Plasma Kallikrein and Thrombin

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Received November 6, 1995; Revised Manuscript Received February 28, 1996⊗

ABSTRACT: As discussed in the accompanying paper [Markland, W., Ley, A. C., & Ladner, R. C. (1996) *Biochemistry 35,* 8045−8057], we generated libraries from the first Kunitz domain of human lipoprotein-associated coagulation inhibitor (LACI-D1) using multivalent M13 III display and derived potent inhibitors of human plasmin (PLA) by iterative variegation and selection. Here, we show that high-affinity, high-specificity binders to human plasma kallikrein (pKAL) and human thrombin (THBN) can be obtained starting from the identical library and employing the same iterative variegation procedures used to obtain PLA inhibitors. Lib#1 (allowing 31 200 variants involving five positions near the P1 residue of LACI-D1) and its pKAL-biased derivative, Lib#4 (allowing an additional 1600 variants at residues 31, 32, 34, and 39), were screened against pKAL, yielding potent inhibitors. One of these, EPI-K401, has $K_i = 284$ pM, very high specificity, and excellent stability. We used information from Lib#4 selectants to design Lib#5 (allowing 1.5×10^6 amino-acid sequences involving nine varied positions) from which we obtained an inhibitor (EPI-K503) having high affinity for pKAL ($K_i = 40$ pM) and retaining the high specificity of EPI-K401. When we screened Lib#1 and its THBN-tailored derivative, Lib#6, against THBN, we obtained a different and very homogeneous population of selected molecules. The purified proteins derived from Lib#6 selectants bound to THBN-agarose beads but did not inhibit proteolytic activity of THBN, suggesting that these selectants bind to a site on THBN other than the catalytic site. Thus, a single large combinatorial library can serve as a source to obtain highly specific, high-affinity binding molecules for each of several targets. Furthermore, the results with THBN show that the binding of Kunitz domains to other proteins is not limited to the catalytic sites of trypsin-homologous proteases.

The Kunitz domains comprise a large family of small (∼58 amino acids), highly stable molecular units. The prototypical Kunitz domain, bovine pancreatic trypsin inhibitor (BPTI, also known as aprotinin or Trasylol),[1] inhibits trypsin by formation of a particularly tight complex in which 14 residues of BPTI contact 25 residues of trypsin in the catalytic site region. We and others have shown that inhibitors of trypsin-homologous proteases can be engineered using bacteriophage display of Kunitz domains (Markland *et al*., 1991, 1996; Ladner & Markland, 1995; Roberts *et al*., 1992a,b; Dennis & Lazarus, 1994a,b). Using monovalent phage display and site-directed mutagenesis, Dennis *et al*., (1995) selected variants of the Kunitz domain of amyloid precursor protein I (APP-I-D1) for binding to human plasma kallikrein (pKAL) and obtained highly similar sequences to those reported here. In the preceding paper, we described an iterative approach to library construction and screening (Markland *et al*., 1996). We displayed the first Kunitz domain of the human serum protein lipoprotein-associated coagulation inhibitor (LACI-DI) as an amino-terminal fusion to the III protein of phage M13 and generated combinatorial libraries of the domain in which we varied thirteen positions in the region corresponding to the trypsin−BPTI interface. We screened the libraries for components that bound to immobilized human plasmin (PLA) and obtained an inhibitor of PLA that binds with high specificity and $K_i = 87$ pM and differs from human LACI-D1 by only seven amino acids (Markland *et al*., 1996). Here, we show that the same LACI-D1 library can yield high-affinity, high-specificity binders to human plasma kallikrein (pKAL) and human thrombin (THBN). We then used information from selected pKAL binders to design a pKAL-tailored library (Lib#5) from which we obtained an inhibitor of greater potency (40 pM) and high specificity.

Human plasma kallikrein [pKAL, EC.3.4.21.34; reviewed by Bhoola *et al*. (1992a)] is involved in the regulation of several important physiological pathways including: contact-activated (intrinsic pathway) coagulation, fibrinolysis, hypotension, and inflammation (Bhoola *et al*., 1992b). Since inappropriate pKAL activity is thought to mediate several diseases, a potent, highly specific inhibitor of pKAL is an important therapeutic candidate (Bhoola *et al*., 1992b).

BPTI (aprotinin), also known as a "kallikrein inhibitor", has been described as a strong pKAL inhibitor with $K_i =$ 320 pM (Auerswald *et al*., 1988). Other reports differ: Fritz and Wunderer (1983) and Berndt *et al*. (1993) indicate that

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⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1996.

[1] Abbreviations: Ap, ampicillin; APP-I-D1, Kunitz domain of amyloid precursor protein-I; BPTI, bovine pancreatic trypsin inhibitor; F.VII$_a$, coagulation Factor VII$_a$; F.XI$_a$, coagulation Factor XI$_a$; FIR, fraction of input recovered; hNE, human neutrophil elastase; KAB, kallikrein assay buffer; LACI-D1, first Kunitz domain of lipoprotein-associated coagulation inhibitor; NKD, natural Kunitz domain; PAB, plasmin assay buffer; PEG, polyethylene glycol; pfu, plaque forming units; pIII, M13 protein III; pKAL, human plasma kallikrein; PLA, human plasmin; RSB, reaction stop buffer; RT, room temperature; THBN, human thrombin; Vg, variegated; vgDNA, variegated DNA; w.t., wild-type; %RA, percent residual activity; ::, fusion (gene or protein). Trasylol is a trademark of Bayer, AG.

S0006-2960(95)02629-8 CCC: $12.00

Table 1: Libraries of LACI-D1 from Which pKAL Binders Were Isolated

name	DNA in 10−21[a]	DNA in 31−39[b]	no. of amino-acid sequences allowed[c]	no. of DNA sequences allowed	no. of transformants
Lib#1	Vg#1[d,e]	w.t.	31 200	65 536	5.3×10^5
Lib#4	Vg#1 selected for pKAL	Vg#2[f]	5.0×10^7 [g]	2.7×10^8 [g]	3.9×10^7
Lib#5	Vg#4[h]	to encode EEFSYGGCG	1.5×10^6	4.2×10^6	1.2×10^6

[a] P1 region. [b] Second-loop region. [c] Assuming that TAG is translated as Q in Sup^E strains. [d] vgDNA comprises codons 13−19, 5′-cNt tgt aaa gSt NNt NNS NNg-3′, so that 65 636 DNA sequences encode 31 200 amino-acid sequences. [e] Mixed bases (all intended to be equimolar): N = ACGT, B = CGT, D = AGT, H = ACT, V = ACG, K = GT, M = AC, R = AG, S = CG, W = AT, Y = CT. Single-letter amino-acid codes: A = Ala, C= Cys, D = Asp, E = Glu, F = Phe, G = Gly, H = His, I = Ile, K = Lys, L = Leu, M = Met, N = Asn, P = Pro, Q = Gln, R = Arg, S = Ser, T = Thr, V = Val, W = Trp, Y = Tyr. [f] vgDNA comprises codons 31−39, 5′-Sag Sag ttc NNS tac ggt ggt tgt NNS-3′, so that 4096 DNA sequences encode 1600 amino-acid sequences. [g] Assuming complete representation of both Vg#1 and Vg#2. [h] vgDNA comprises codons 10−21, 5′-RaS RNt ggt NNt tgt aRa gSt RNt cNS cNS cgt tKS-3′, so that 4.19×10^6 DNA sequences encode 1.0×10^6 amino-acid sequences.

the K_i between pKAL and BPTI is 30 nM, while Scott *et al*. (1987) report 320 nM. BPTI(G36S) has a K_i for pKAL of over 500 nM (Berndt *et al*., 1993). BPTI is a better inhibitor of human tissue kallikrein (Calbiochem); we measure the K_i to be about 6 nM (data not shown).

Aprotinin reduces perioperative bleeding (Royston *et al*., 1987; Bidstrup *et al*., 1989), and it is widely accepted (Royston, 1994) that this effect is due to inhibition of one or more proteases. Nevertheless, it is not clear which protease inhibition is responsible for the effect (Wildevuur *et al*., 1989). Wachtfogel *et al*. (1993) report that "aprotinin inhibits the contact-, neutrophil-, and platelet-activation systems during simulated extracorporeal perfusion". Wachtfogel *et al*. (1995) report that specific inhibitors of the contact pathway can reduce complement and neutrophil activation during simulated extracorporeal circulation. Cumming (1994), using a significantly higher dose than Puttermann (1989), showed that BPTI (although not very potent against pKAL) can greatly reduce undesirable effects associated with excess kallikrein activity in induced sepsis in animals. Increasing affinity for pKAL is likely to improve efficacy.

We desired to determine whether a Kunitz domain could be engineered to bind and inhibit THBN. Bode *et al*. (1989) and Le Bonniec *et al*. (1992, 1993) suggest that a Kunitz domain could not reach the active site of THBN without displacing a loop that is inserted (relative to chymotrypsin) after residue 60. We found a class of Kunitz domains that bind THBN-agarose beads, but there is no inhibition of free THBN by phage-free proteins. The most likely explanation is that the selected domains bind to a site on THBN other than the catalytic site.

EXPERIMENTAL PROCEDURES

Library Constructions. Lib#1 is the same as Lib#1 described by Markland *et al*. (1996). We made Lib#4 (Table 1) by ligating DNA encoding the variegation scheme Vg#2 into DNA prepared from a pool containing about 300 different phage that had been selected from Lib#1 through two rounds for binding pKAL-agarose. We estimate that Lib#4 contains between 3×10^5 and 5×10^5 variants. Lib#5 was prepared in a manner very similar to the preparation of Lib#3 (Markland *et al*., 1996) using DNA from LACI-D1.III-(K401) and Vg#5 variegated DNA. The library titer was 1×10^{11} plaque forming units (pfu) per mL. Lib#6 was prepared from a pool containing about 300 different phage from Lib#1 which had been selected for binding to THBN by introduction of Vg#2; we estimate the Lib#6 contains between 3×10^5 and 5×10^5 variants.

Screening and Characterization of Selectants. "Slow screen", "quick screen", "display-phage purification", and "display-phage binding specificity" protocols were defined previously (Markland *et al*., 1996). We prepared pKAL beads by coupling pKAL (Calbiochem, 420302) to Reacti-Gel (6X) support (Pierce, 20259) as described (Markland *et al*., 1996) and used commercially available PLA beads (Calbiochem, 527802) and THBN beads (Calbiochem, 605204).

Protein Production and Purification. To produce the pKAL inhibitor protein EPI-K401 for characterization studies, we constructed the *Pichia pastoris* production strain PEY-47 as described for PEY-73 (Markland *et al*., 1996). We grew cultures of PEY-47 at 30 °C with agitation in a minimal complex medium (Barr *et al*., 1992) buffered at pH 3 and supplemented daily with 1% (v/v) MeOH. After 3 days of growth, we harvested the cultures, removed cells by centrifugation (8,000*g*, 15 min), and filtered the culture supernatant twice through 0.45 μ filters and once through a 0.2 μ filter (Costar Corporation, Cambridge, MA). We next performed a 30 K ultrafiltration on the 0.2 μ filtrate using a Minitan apparatus equipped with four 30 000 NMWL filter plates (PLKCC0MP04, Millipore Corporation, Bedford, MA). To purify EPI-K401 from the 30 K ultrafiltrate, we adjusted the pH of the solution to 3.5 and then applied it to a 1 mL bed volume of MP-50S (Bio-Rad Laboratories, Hercules, CA) cation exchange column. We eluted the column using 20 mL volumes of 50 mM NH_4OAc at pH steps of 3.5, 4.5, 5.5, and 6.9. EPI-K401 eluted in the pH 6.9 fraction. We reduced the pH of the eluate from pH 6.9 to 3.5, reapplied it to the cleaned, regenerated MP-50S column, and eluted the column with a pH step gradient between pH 3.5 and 6.0 using 10 mL volumes of 50 mM NH_4OAc in 0.5 pH unit steps. In the final pH step, we eluted EPI-K401 from the column at pH 6.9. We removed volatile buffer components from the ion exchange purified EPI-K401 by lyophilization and dissolved the dried protein in water at a final concentration of 1.4 mg/mL (*ca*. 200 μM). The final protein stock was >95% pure EPI-K401 as determined by inspection of silver-stained PAGE gels, and the overall yield of the purification procedure was about 44%.

We produced the pKAL inhibitor EPI-P503 and THBN-binding proteins derived from Lib#6 isolates using the *Saccharomyces cerevisiae* expression system and purified proteins from culture media with trypsin-agarose (Pierce, 20230) or thrombin-agarose (Calbiochem, 605204), respectively, as described (Markland *et al*., 1996).

Protein Characterizations. We characterized purified EPI-K401 and EPI-K503 for their pKAL inhibition properties as

previously described for the plasmin inhibitors (Markland *et al*., 1996) with the following changes. In all assays of pKAL (American Diagnostica Inc., 473) activity, we used the fluorogenic substrate H-Pro-Phe-Arg-7-amido-3-methylcoumarin (Novabiochem, 03-37-1547; K_M *ca.* 290 μM). For active site titration of pKAL using aprotinin, the reactions contained 1.5 μM pKAL in kallikrein assay buffer (KAB: 150 mM NaCl, 20 mM Tris·HCl pH 7.5, 1 mM EDTA, 0.1% PEG).

To determine K_i for inhibition of pKAL by EPI-K401, we incubated 12.9 nM pKAL and inhibitor in 150 μL of KAB for 90 min at room temperature prior to adding 16 μM substrate (0.06 K_M). Following a further 10 min incubation, we stopped substrate hydrolysis by transferring 100 μL of the reaction mixture to 2.0 mL of reaction stop buffer (RSB: citrate-phosphate buffer, pH 3, containing 0.1% Triton X-100). To measure K_i for inhibition of pKAL by EPI-K503, we incubated 1.0 nM pKAL with inhibitor in 200 μL of KAB for 150 min at room temperature prior to adding 45 μM substrate (0.16K_M) and stopped substrate hydrolysis after 10 min by adding 190 μL of the reaction mix to 2.0 mL of RSB.

We measured kinetic on rates (k_{on}) using the procedure described (Markland *et al*., 1996) with all components in KAB and 45 μM substrate. To determine k_{on} for EPI-K401, we reacted 9.65 nM pKAL with 11 nM inhibitor; for EPI-K503, concentrations were 1.0 and 1.5 nM, respectively.

We performed stability and specificity assays, fluorescence measurements, calculations, and curve fitting as previously described (Markland *et al*., 1996). For measurements of plasmin inhibition, we incubated plasmin (Calbiochem, 527642) at 1 nM in plasmin assay buffer (PAB: 150 mM NaCl, 50 mM Tris·HCl, pH 7.5, 0.1% PEG, 0.05% Triton X-100) and added substrate (Sigma, S-0763) at 50 μM.

THBN Inhibition Assays. We assayed for inhibition of THBN catalytic activity by thrombin-binding proteins using two assay systems. For the fluorogenic substrate hydrolysis assay, we incubated protein with 0.54 nM THBN in 100 μL PAB for 30 min and then transferred the reaction to 2 mL of PAB containing 5 μM fluorogenic substrate (N-*t*-Boc-Val-Pro-Arg-7-amido-3-methylcoumarin, Sigma, B9385). We determined relative rates of THBN catalytic activity from the rates of increase of sample fluorescence. As a second assay for THBN activity, we used a plasma coagulation assay (Sigma Diagnostics, C-7916) according to the manufacturer's instructions. We used the synthetic inhibitor Thromstop (American Diagnostica Inc., 505) as a positive control for THBN inhibition.

RESULTS

pKAL

Screening of Lib#1 and Lib#4 against pKAL. As was the case for the development of PLA inhibitors (Markland *et al*., 1996), we performed the first iteration of library construction and screening in two steps. We started by using the same Lib#1 that served as the starting point in the development of EPI-P302 (Markland *et al*., 1996). Three rounds of quick screen of Lib#1 for variants with affinity for pKAL-agarose yielded about 300 selectants. We determined DNA sequences for 16 different isolates; of these 11 were unique (Table 2), indicating that the pool probably contains about 200 different sequences. Table 3 shows the frequency of observed amino-acid types at each of the varied positions. There is substantial enrichment of specific amino acids at three of the five varied positions: 13 (P or R), 18 (H), and 19 (L).

Table 2: LACI-D1 and Variants That Bind pKAL Derived from Lib#1

identifier	111111111122[a] 012345678901	ΔL[b]	ΔC[c]	N_{occur}[d]
LACI-D1	ddgpckaimkrf	0	3	-
K1con[e]	"""P""AAHL""	3	0	-
K101	"""R""G---""	5	2	3
K102	"""-""-I--""	3	1	2
K103	"""-""-I-M""	2	2	1
K104	"""-""-N--""	3	1	1
K105	"""-""-SIP""	3	3	1
K106	"""-""-Y--""	3	1	1
K107	"""-""G---""	4	1	1
K108	"""-""G-IP""	4	3	2
K109	"""R""---P""	4	2	1
K110	"""R""-S--""	4	2	2
K111	"""R""GP--""	5	3	1

[a] Position in Kunitz domain by analogy to BPTI. Below are the single-letter codes for (1) LACI-D1 (w.t.), (2) K1con, the consensus of the selected sequences, and (3) the selected sequences K101−K111. Residues not varied are shown as """. Those that were varied and have the consensus amino acid are shown as "−". [b] Differences from LACI-D1. [c] Differences from K1con, the consensus of Lib#1 pKAL selectants. [d] Number of times sequence was observed. [e] K1con is the consensus of sequences K101−K111.

In the second step, we added the 1600-fold diversity of Vg#2 into the amplified phage population we obtained from round three of the quick screen of Lib#1 to make Lib#4. Starting with an input of 1.7×10^{10} pfu, we obtained a small number (*ca*. 15−20) of isolates from the third round of a quick screen of Lib#4 against immobilize pKAL. Deduced amino-acid sequences of 10 isolates selected from Lib#4 for pKAL binding are shown in Table 4 as K401-K407.[2] K4con is the consensus of these sequences and is identical to K401, which occurred four times. The consensus is H_{13}ckANHQ_{19}−E_{31}EfSyggcG_{39} (lower case residues were not varied). Table 5 shows the frequency of amino-acid types observed at each varied position in the Lib#4 isolates that we sequenced. Even allowing for the small sample size, significant enrichment of specific amino acids or sets of amino acids is observed at all nine positions that were varied in Lib#4.

Affinity and Specificity of Selected pKAL-Binding Display Phage. We determined the relative affinities of various display phage for pKAL immobilized on agarose by measuring FIR for a single round of slow screen. These data are shown in Table 6. All four of the tested isolates obtained from the first iteration of screening (two from Lib#1 and

[2] LACI-D1 variants selected for binding pKAL are designated K*j*mm, where *j* is the library number and mm is the isolate number from that library. THBN selectants are designated T*j*mm. The free proteins comprise $E_{-2}A_{-1}$::LACI-D1 (K*j*mm) or $E_{-2}A_{-1}$::LACI-D1 (T*j*mm). In each case, the residues of the LACI-D1 domain are numbered as shown in Table 2 of Markland *et al*. (1996).

Table 3: Amino-Acid Types Observed at Variable Positions of Lib#1

position[a]	allowed in Vg#1	observed[b]	not observed
13	LPHR	P:9 R:7	LH
16	AG	A:9 G:7	−
17	ACDFGHILNPRSTVY	A:7 I:3 S:3 PNY	CDFGHLRTV
18	ACDEFGHIKLMNPQRSTVWY	H:12 I:3 Q	ACDEFGKLMNPRSTVWY
19	AEGKLMPQRSTVW	L:10 P:4 V M	AEGKQRSTW

[a] Position in Kunitz domain, by analogy to BPTI. [b] Single-letter identifier followed by number of times it was observed, "1"s omitted.

Table 4: LACI-D1 and Variants That Bind pKAL Derived from Lib#4

identifier	111111111122[a] 012345678901	333333333[a] 123456789	ΔL[b]	ΔC[c]	N_{occur}[d]
LACI-D1	ddgpckaimkrf	eefiyggce	0	6	-
K4con[e]	ddgHckANHQrf	EEfSyggcG	6	0	4, as K401
K401	"""-""----""	--"-""""-	6	0	4
K402	"""-""----""	--"T""""-	6	1	1
K403	"""P""---L""	--"-""""-	5	2	1
K404	"""-""----""	-Q"T""""A	7	3	1
K405	"""-""-SLP""	--"I""""-	5	4	1
K406	"""-""-SLP""	--"I""""E	4	5	1
K407	"""-""GA-L""	--"I""""E	5	5	1

[a] Positions in Kunitz domain by analogy with BPTI. Residues shown below as in Table 2. K4con is consensus of K401−K407. Residues shown in upper case were varied, and those shown in lower case were not. [b] Differences from LACI-D1. [c] Differences from K4con. [d] Number of times sequence was isolated. [e] K4con is the consensus of sequences K401−K407.

two from Lib#4) show substantial improvement in affinity for pKAL relative to either the parental display phage (LACI-D1.III) or a phage clone displaying the BPTI::gene III protein fusion (BTPI.III). FIR for the Lib#1 and Lib#4 isolates are all between 2 and 3 orders of magnitude greater than the FIR obtained with either of the control phage, and affinities of Lib#4-derived phage appear higher than those of Lib#1-derived phage.

The display phage isolate LACI-D1(K401) appears to have the highest affinity for pKAL of the four isolates tested in Table 6. We further characterized this isolate for specificity of binding to proteases immobilized on agarose beads. Relative affinities for binding (measured as FIR) by the phage LACI-D1.III and LACI-D1(K401).III to the three related human serum proteases pKAL, PLA, and THBN immobilized on agarose beads are compared in Table 7. Relative to LACI-D1.III phage, the affinity for pKAL of the pKAL-selected phage LACI-D1(K401).III has increased 196-fold while the affinities for THBN and for PLA show little change. The affinity of LACI-D1 is least for pKAL and greatest for PLA. The affinity of LACI-D1(K401) is highest for pKAL.

Expression and Characterization of EPI-K401. We used the *P. pastoris* expression system to produce the protein, EPI-K401, displayed by the phage isolate LACI-D1(K401).III and purified the protein from the culture medium. We characterized the purified protein for its pKAL inhibition properties, specificity, and stability.

Purified *P. pastoris*-produced EPI-K401 has potent inhibitory activity toward pKAL, with $K_i = 284$ pM [determined in the way given in Markland *et al*. (1996)]. We measured the k_{on} of EPI-K401 and pKAL [determined in the way given in Markland *et al*. (1996)]; the least-squares best value is $k_{on} = 0.21 \times 10^6$/M/s, giving $k_{off} = 56. \times 10^{-6}$/s.

We determined the level of inhibition of several human proteases by EPI-K401. For all of the enzymes except pKAL, IC_{50} is much greater than the concentration of enzyme; conditions where $IC_{50} = K_i$. The K_i for EPI-P401 inhibition of PLA (~120 nM) is ~430-fold higher than the K_i for pKAL inhibition; [PLA] was 13 nM. All other proteases tested have much higher K_i values: human chymotrypsin (K_i = ~600 nM, [chymotrypsin] = 20 nM), hNE (K_i = ~12 μM, [hNE] = 8.5 nM), Factor X_a (K_i > 100 μM, [Factor X_a] = 11 nM), urokinase (K_i > 100 μM, [urokinase] = 75 nM), and THBN (K_i > 100 μM, [THBN] = 0.5 nM). For Factor Xa, urokinase, and THBN, there is no inhibition at the highest amount of EPI-K401 tested (15 μM).

Figure 1 presents measurements of the resistance of EPI-K401 to denaturation by extremes of temperature (panel A), pH (panel B), and oxidation (panel C). The pKAL-inhibition activity the inhibitor is essentially unaffected (>85% residual inhibitory activity) by incubation at 96 °C for 30 min, 18 h incubation at pH of less than 10, and 30 min incubation in the presence of more than 50-fold molar excess of the oxidant chloramine T.

Iteration of Variegation and Selection for pKAL. We used the information about pKAL-binding sequence determinants obtained from Lib#1 and Lib#4 to design (see Discussion) the variegation scheme Vg#5 which provides extended variation in the P1 region (Schechter & Berger, 1968). To construct Lib#5, we incorporated the DNA encoding Vg#5 into LACI-D1(K401).III RF DNA. The resulting library contains the extended variation in the P1 region and EPI-K401 sequences at positions 31−39 (see Table 1). We then screened Lib#5 against pKAL-agarose beads using three rounds of slow screen. FIR increased at each round so that the FIR of round three (1.4×10^{-3}) was about 100-fold greater than that from the first round (1.1×10^{-5}).

Sequences and pKAL Binding of Lib#5 pKAL-Binding Selectants. Eleven unique amino acid sequences from thirteen round 3 selectants from Lib#5 are shown in Table 8; Table 9 shows which amino-acid types were observed at each of the varied positions. The consensus sequence (K5con) in the P1 region is D_{10}(D/G)gRcRGAHPrW_{21} (lower case amino acids were not varied; D and G occur almost equally often at position 11). Significant levels of enrichment (present in nine or more isolates, *i.e.*, >70%) for specific residues are seen at D_{10}(12/13), R_{15}(13/13), A_{17}(10/13), H_{18}(9/13), P_9(9/13), and W_{21}(9/13). At position 16, G(8) is slightly preferred to A(5). No strong enrichment for a single residue is observed at position 11, although G and D both occur four times. The K501 and K503 sequences were each observed twice, all others were observed once.

Table 5: Amino-Acid Types Observed at Variable Position in Lib#4 Selectants

position	allowed in Vg#1 or Vg#2	observed[a]	not observed
13	HPRL	H:9 P	RL
16	AG	A:9 G	−
17	ACDFGHILNPRSTVY	N:7 S:2 A	CDFGHILPRTVY
18	ACDEFGHIKLMNPQRSTVWY	H:8 L:2	ACDEFGIKMNPQRSTVWY
19	AEGKLMPQRSTVW	Q:6 L:2 P:2	AEGKMRSTVW
31	EQ	E:10	Q
32	EQ	E:9 Q	−
34	ACDEFGHIKLMNPQRSTVWY	S:5 I:3 T:2	ACDEFGHKLMNPQRVWY
39	ACDEFGHIKLMNPQRSTVWY	G:7 E:2 A	CDFHIKLMNPQRSTVWY

[a] Observed amino-acid type followed by number of times observed, "1"s omitted.

Table 6: Binding of Phage to pKAL-Agarose As Measured by FIR

phage[a]	FIR[b]	FIR/FIR(LACI-D1.III)
LACI-D1.III	4.2×10^{-6}	$\equiv 1.0$[c]
BPTI.III[d]	2.5×10^{-5}	6
LACI-D1(K101).III[e]	3.2×10^{-3}	761
LACI-D1(K102).III	2.2×10^{-3}	524
LACI-D1(K405).III	3.9×10^{-3}	928
LACI-D1(K401).III	8.7×10^{-3}	2471

[a] All phage are complete M13 modified in the single *iii* gene by insertion of LACI-D1, BPTI, or a variant of LACI-D1. [b] FIR, fraction of input phage recovered (Markland *et al*., 1996). [c] The binding of LACI-D1.III phage is taken as unity. [d] BPTI.III is an M13 derivative having BPTI fused to the amino terminus of mature III protein. [e] Variants of LACI-D1 are indicated by the isolation number (Tables 2 and 4). LACI-D1(K101) is LACI-D1 with the alterations P13R, A16G, I17A, M18H, and K19L.

Table 7: Phage Binding (Measured As FIR) of LACI-D1.III and LACI-D1(K401).III to the Three Related Human Serum Proteases pKAL, PLA, and THBN Immobilized on Agarose Beads

protease	$A =$ FIR(LACI-D1.III)	$B =$ FIR(LACI-D1(K401).III)	*A/B*
pKAL	5.1×10^{-6}	1.0×10^{-3}	196
PLA	2.4×10^{-4}	8.2×10^{-4}	3.4
THBN	1.8×10^{-5}	2.7×10^{-5}	1.5

The sequence of EPI-K503 is close to K5con, has the fewest changes from the parental LACI-D1 sequence and was observed twice among the Lib#5 selectants. We used our *S. cerevisiae* production and purification procedures (Markland *et al*., 1996) to produce small amounts of purified EPI-K503 for analysis. The *S. cerevisiae*-produced protein EPI-K503 differs from EPI-K401 at five positions and inhibits pKAL with $K_i = 40$ pM, a 7-fold improvement over EPI-K401. EPI-K503 has k_{on} for pKAL equal to 1.5×10^6/M/s (giving $k_{off} = 61. \times 10^{-6}$/s). We measured the K_i of EPI-K503 for PLA (the enzyme other than pKAL most inhibited by EPI-K401) to be ~20 nM. The ratio K_i(PLA)/K_i(pKAL) is slightly greater for EPI-K503 than for EPI-K401, but this may not be significant.

THBN

Sequences of THBN-Binding Lib#6 Selectants. We screened Lib#1 through one slow and three quick rounds for binding to THBN-agarose beads and obtained a pool containing about 300 selectants. This pool was amplified, and Lib#6 was produced by introducing Vg#2 into this population.

Lib#6 was passed through 3 rounds of quick screen from which we obtained about 800 selectants. The pool of display-phage selectants from the third round of Lib#6 quick screen was amplified, and individual isolates were then picked for further analysis. Table 10 gives twelve sequences of isolates from Lib#6 selected for binding to THBN. T6con, the consensus, is R_{13}ckGDGG$_{19}$−E$_{31}$EfG$_{34}$yggcT$_{39}$ and is identical to T601. Overall, the twelve sequences comprise an extremely homogeneous family of proteins. Only one sequence (T610) differs from the consensus in the P1 region, although the variation among the sequences is more extensive at positions 31−39.

Properties of Isolated Display Phage and Purified Proteins. We prepared stocks of the purified phage isolates LACI-D1(T601) and LACI-D1(T602) for analyses of binding affinity and specificity. On the basis of FIR, the display phage LACI-D1(T601) and LACI-D1(T602) show 480- and 320-fold, respectively, better binding to THBN-agarose beads than do LACI-D1.III phage. Table 11 shows the FIR of LACI-D1(T601).III and LACI-D1.III phage for four proteases. The T601 variant is highly specific for THBN and has very little affinity for the proteases pKAL, PLA, and bovine trypsin, all of which have less substrate specificity than does THBN.

We produced EPI-T601 and EPI-T602 as free proteins in *S. cerevisiae* culture medium and purified the proteins from the yeast culture medium using THBN-agarose beads. Even though the free proteins can be effectively purified using THBN-agarose, neither protein demonstrated any inhibition of THBN catalytic activity assayed either by fluorogenic substrate hydrolysis or in a plasma coagulation assay.

DISCUSSION

pKAL

Design of Lib#1 and Lib#4. The designs of Vg#1 and Vg#2 have been discussed (Markland *et al*., 1996). The variegation scheme accommodates the sequence requirements of Kunitz domains and produces a large number of surfaces without direct reference to any particular target. Lib#4 differs from Lib#2 (Markland *et al*., 1996) only in that it was made from DNA selected to improve binding to pKAL instead of to PLA.

Lib#1 and Lib#4 Selectants. Quick screening for three rounds produced a small pool (~300) of selected sequences that probably contains ~200 different sequences. A sample of these is shown in Table 2. Combining the observed amino-acid types at each position gives 288 sequences while Lib#1 allowed 31 200 sequences. Thus, the apparent complexity has been reduced by about 150-fold. Among the Lib#1 selectants, no isolate we sequenced contained the consensus, P$_{13}$ckAAHL$_{19}$, although four sequences differ by only one amino acid. No selected sequence differed from the consensus by more than three amino acids. Four

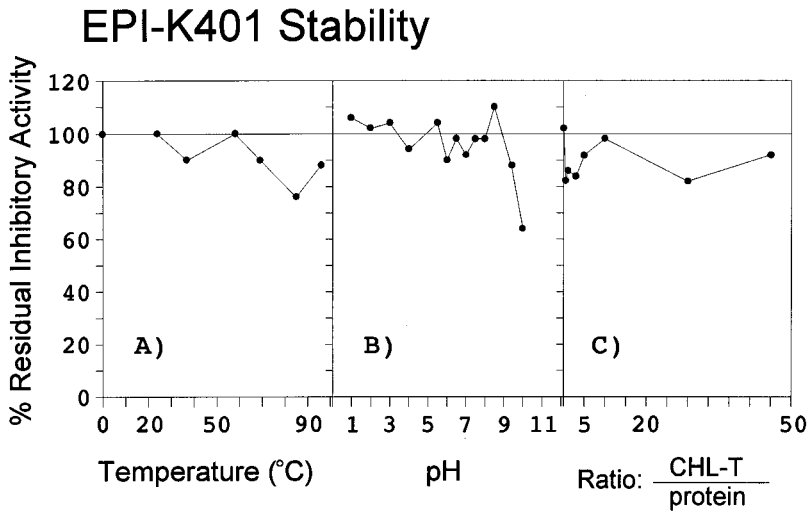

FIGURE 1: Stability of EPI-K401. Percent residual pKAL-inhibition activity of EPI-K401 is shown following the various treatments shown. Panel A shows the percent residual inhibitory activity (measured at RT) of EPI-K401 after incubation of the inhibitor for 30 min at temperatures up to 95 °C. Panel B shows percent residual inhibitory activity (at RT) for EPI-K401 after incubation for 18 h at 37 °C at the indicated pH. Panel C shows a percent residual inhibitory activity of EPI-K401 after incubation for 30 min at RT in the presence of various amounts of chloramine T.

Table 8: LACI-D1 and Variants That Bind pKAL Selected from Lib#5

identifier	111111111122[a] 012345678901	333333333[a] 123456789	ΔL^b	ΔC^c	ΔK^d	$N_{occur}{}^d$
LACI-D1	ddgpckaimkrf	eefiyggce	0	9	6	-
K5con[f]	DDgRcRGAHPrW	EEfSyggcG	9	0	6	0
K501	-G"-"-----"-	""""""""	10	1	7	2
K502	-V"-"-----"-	""""""""	10	1	7	1
K503	--"P"-A---"-	""""""""	7	2	5	2
K504	--"H"---L-"-	""""""""	9	2	6	1
K505	-V"-"---Q-"F	""""""""	9	3	7	1
K506	--"S"-A--L"-	""""""""	8	3	5	1
K507	--"P"----L"F	""""""""	7	3	5	1
K508	-S"-"--N-Q"F	""""""""	9	4	4	1
K509	-G"-"-AIQ-"-	""""""""	9	4	7	1
K510	EG"S"-A--Q"-	""""""""	10	5	6	1
K511	-S"N"--NL-"F	""""""""	9	5	6	1
KALI-DY[g]	eDGHCRAAHPrw	apfYyggcG	11	6	-	-

[a] Position in Kunitz domain by analogy to BPTI. Below are sequences selected from Lib#5 with same convention as in Table 2. [b] Amino-acid differences from LACI-D1. [c] Amino-acid differences from K5con, the consensus of sequences K501−K511. [d] Amino-acid differences from EPI-K401. [e] Number of times the sequence was isolated. [f] K5con is the consensus of sequences K501−K511. [g] Sequence from Dennis *et al.* (1995).

sequences occurred more than once, suggesting that the pool contains fewer than 200 different sequences and that these are clustered around the observed consensus. The selection at position 17 excludes charged and aromatic groups (Table 3). At positions 18 and 19, the selection is fairly tight.

Introduction of additional variation in the loop (positions 31−39) underlying the P1 region to make Lib#4 resulted in a somewhat different consensus sequence (K4con, Table 4) in the P1 region following selection for pKAL binding: H_{13}-ckANHQ$_{19}$. In only two of the five varied positions in the P1 region was the K1con residue retained: position 18, where

Table 9: Types of Residues Selected from Lib#5 at Each Variable Position

position	allowed in Vg#4[a]	observed	not observed
10	DEKN	D:12 E	NK
11	ADGINTVS	D:5 G:4 V:2 S:2	AITN
13	ACDFGHILNPRSTVY	R:6 P:3 S:2 H N	ACDFGILTVY
15	KR	R:13	K
16	AG	G:8 A:5	−
17	ADGINTVS	A:10 N:2 I	DGSTV
18	HLPQR	H:9 L:2 Q:2	PR
19	HLPQR	P:9 L:2 Q:2	HR
21	CFLW	W:9 F:4	CL

[a] As in Table 3.

the strong selection for H was repeated, and position 16, where A is now more strongly selected over G.

Positions 13, 17, and 19 (in the P1 region) exhibited changes in the consensus residue between Lib#1 and Lib#4 selectants. The strong preference of H_{13} among the Lib#4 selectants represents a large change from the case found for Lib#1 selectants where P and R were nearly equally common and H was not found. In natural Kunitz domains (NKD), H at position 13 has not been observed.

At position 17, N is fairly strongly selected; N is rare at position 17 in NKDs and was seen only once in Lib#1 selectants. At position 19, Q is moderately selected (6/10); Q was a minor component in the Lib#1 selectants.

At position 31, only E is selected, no Q. Since this is on the edge of the molecular interface, there seems to be positive selection for a negatively-charged side group. At position 32, E is strongly selected (9/10) over Q. Residue 192 in pKAL is K. In the BPTI−trypsin complex, Q_{192} comes within 6 Å of V_{34} of BPTI (the closest trypsin-contacting residue to $E_{31}E_{32}$). Perhaps there is an interaction between $E_{31}E_{32}$ side groups of EPI-K503 and K_{192} of pKAL.

At position 34, only S, T, and I occur even though all twenty types were allowed. S and T have hydroxyl groups in their side group. Two of the three I_{34}-containing isolates have the wild-type (w.t.) residues at all of the supposedly varied positions in the second loop and may come from phage that escaped modification. These data suggest that

Table 10: Partial Deduced Amino-Acid Sequences of Phage Selected for Binding to THBN from Lib#6

identifier	111111111122[a] 012345678901	333333333[a] 123456789	ΔL[b]	ΔC[c]
LACI-D1	ddgpckaimkrf	eefiyggce	0	7
T6con	ddgRckGDGGrf	EEfGyggcT	7	0
T601	"""-""----""	--"-""""-	7	0
T602	"""-""----""	-Q"-""""-	8	1
T603	"""-""----""	--"-""""N	7	1
T604	"""-""----""	--"-""""R	7	1
T605	"""-""----""	--"D""""N	7	2
T606	"""-""----""	Q-"D""""-	8	2
T607	"""-""----""	--"D""""R	7	2
T608	"""-""----""	Q-"Y""""K	8	3
T609	"""-""----""	-Q"T""""L	8	3
T610	"""L""A---""	--"S""""G	6	4
T611	"""-""----""	QQ"Q""""L	9	4
T612[d]	"""-""----""	Q-"P""""C	9	4

[a] Position in Kunitz domain by analogy to BPTI. Residues below are shown with same convention as in Table 2. [b] Amino-acid differences from LACI-D1. [c] Amino-acid differences from T6con, the consensus of T601−T612. [d] This variant also carries the mutation C30G.

Table 11: Binding of LACI-D1(T601).III and LACI-D1.III Phage to Various Proteases

protease[a]	$A =$ FIR(LACI-D1(T601).III)	$B =$ FIR(LACI-D1.III)	A/B
THBN	8.2×10^{-3}	1.8×10^{-5}	480
pKAL	1.2×10^{-5}	5.1×10^{-6}	2.4
PLA	1.1×10^{-4}	2.4×10^{-4}	0.46
bovine trypsin	5.6×10^{-5}	4.1×10^{-2}	0.0014

[a] Each protease is immobilized on agarose.

side groups having a hydroxyl are preferred at this position. At position 39, only G, E, and A are observed. Further, the two isolates having E also have the w.t. sequence at the other supposedly varied positions and probably come from phage that escaped modification in the second loop.

Design of Lib#5. We used information in the amino-acid sequences selected from Lib#1 and Lib#4 in the design of Vg#4 (Table 1) which variegates the P1 region. Vg#4 was designed with three ideas in mind: (a) limiting variability at strongly selected residues increases the likelihood that new variants will bind the same site on pKAL as did the isolates from Lib#4 and reduces the library size, (b) allowing variation at new positions may generate better binding, and (c) allowing most of the observed types since the number of phage isolated from Lib#1 and Lib#4 was quite small. The consensus of Lib#4 pKAL-binding selectants, $H_{13}-A_{16}N_{17}-H_{18}E_{19}-E_{31}Q_{32}-S_{34}-G_{39}$, shows varying degrees of selection at the varied residues, as discussed.

Vg#4 allows variation at positions 10, 11, 15, and 21, which were fixed in Vg#1. Although residue 10 may not touch the target, a charged group could act at longer range to affect binding. Thus, we allowed residue 10 to be acidic, neutral, or basic. D, E, and N have been observed here. At position 11, we allowed small (G, A), acid (D), neutral hydrophilic (S, T, N), and aliphatic (V, I) types. Of these, V and N have not been observed at position 11 in NKDs. At position 13, H had been strongly selected from (PLHR). Since we were introducing variability at the neighboring positions 10 and 11, we allowed the types shown at position 13 to be sure we have the correct amino acid for the newly selected context.

With reasoning similar to that used in designing Lib#3 for PLA (Markland *et al*., 1996), we allowed only K or R at position 15 and G or A at position 16.

We reduced the diversity at positions 17−19. At position 17, we allowed the set (ITNSVADG) which contains the most commonly selected types selected from Lib#1 and Lib4 for pKAL binding. At position 18, H was strongly selected from all twenty types. We reduced the options to (HLPQR), allowing all types observed except I. At position 19, Q had been fairly well selected from the 13 types encoded by NNg, but L and P also appeared (and predominated in the Lib#1 selectants). We thus allowed the set (QLPHR). We introduced diversity at position 21 to allow the possibility of W because this could increase the exposed hydrophobic area. [Exposed hydrophobic surfaces are often important in protein−protein binding (Clackson & Wells, 1995).] Vg#4 was introduced into DNA from K401 which carries the alterations I34S and E39G.

Table 8 shows the pKAL-selected sequences K501−K511. These differ from LACI-D1 by 7−10 amino acids and from their consensus, K5con, by 1−5 amino acids. They also differ from EPI-K401 by between four and seven amino acids with six amino acids being the average. Table 9 shows the types selected at each varied position. Only position 15 gave a unanimous selection.

At position 10, D is retained in 12 of 13 isolates and the remaining isolate has E; no N- or K-containing sequences were isolated. Position 10 is probably at or beyond the edge of the interface, so the selection of an acidic group is probably for a positive contribution to binding rather than avoidance of a conflict. Q_{192} of trypsin touches T_{11} of BPTI, and it is possible that D_{10} interacts with the side group of K_{192} in pKAL. Among NKDs, D and E are about equally common at position 10 and account for almost half of known sequences, but ten other types have been observed here.

At position 11, D is most common (5/13), but G accounts for 4/13 with V and S occurring twice each. Both D and G are rare at position 11 among NKDs. Dennis *et al*. (1995) found that D_{11} is preferred in APP-I-D1 for high-affinity pKAL binding. The selected pattern suggests that none of the amino-acid types tested contributes strongly to binding and that A, T, I, and N may cause some conflict or just offer no advantage.

At position 13, we allowed 15 types. The selectants contain R:6 P:3 S:2 H:1 N:1. This is a change from the Lib#4 selectants where H was strongly selected from the set (HPRL). Dennis *et al*. (1996) found that H_{13} is preferred to confer pKAL binding on APP-I-D1 derivatives.

At position 15, we allowed only K and R; R is selected uniquely. The strong selection of R is consistent with the binding of BPTI and BPTI(K15R) to pKAL (Scott *et al*., 1987), but the relative effect here is probably smaller. Between BPTI (K_i = 320 000 pM) and BPTI(K15R) (15 000 pM) the improvement is aobut 20-fold, while the improvement between EPI-K401 (K15) (284 pM) and EPI-K503 (R15) (40 pM) is about 7-fold. Since EPI-K401 and EPI-K503 differ at five positions (H13P, K15R, N17A, Q19P,

F21W), we do not attribute all the improvement in binding to the K15R alteration. Dennis and Lazarus (1994b) report K_i[pKAL] for APP-I-D1 and sixteen variants. The best pKAL binder of this group is TF7I-C with $K_i = 1200$ pM. All of the variants that have affinity for pKAL better than 500 000 pM have R_{15}. Thus, the affinity of most Kunitz domains for pKAL can probably be improved by asserting R at position 15. Dennis *et al*. (1995) found that all APP-I-D1 derivatives selected for pKAL binding have R_{15} without exception. To get affinity below nanomolar, however, several other positions need to be optimized for pKAL binding. If other positions have been optimized for pKAL binding, the effect of K15R may be much less than it is when other positions are strongly non-optimal.

At position 16, G is strongly selected over A. This is a change from the Lib#4 selectants and may be related to the shift to R_{15}. Dennis *et al*. (1995) found that A was preferred over G; this could be due to differences between APP-I-D1 and LACI-D1. At position 17, A is selected over N and I, consistent with the Lib#1 selectants and with Dennis *et al*. (1995), but not with the Lib#4 selectants where N was preferred over S and A. At position 18, H is fairly strongly selected, consistent with the selection from Lib#1 and Lib#4 and with Dennis *et al*. (1995). At position 19, P is now selected over L and Q, also consistent with Dennis *et al*. (1995). This is a change from Lib#4 where Q was the most common and Lib#1 where L was most common.

At position 21 (fixed as F in Lib#4), W is selected over F by 9 to 4. Although Y_{21} of BTPI does not touch trypsin, the change to W provides a larger side group with a large hydrophobic surface. In BPTI, the side group of Y_{21} lies between the side group of V_{34} and the main chain of residues 46−48. When this is changed to W, the larger indole side group is likely to extend in the direction of the side group of residue 46 (away from C_{30}). This would place the hydrophobic side group close to the target and close to the side group of residue 19. Thus, W_{21} may contribute directly to pKAL binding.

Despite 37 differences in 58 positions, selection for binding to pKAL causes LACI-D1 and APP-I-D1 to converge to very similar sequences. Table 8 shows a partial sequence of KALI-DY, the highest affinity (15 ± 14 pM) inhibitor reported by Dennis *et al*. (1995). At positions 11, 13, 15−19, 34, and 39, both groups allowed variation. Only at positions 13, 16, and 34 were different amino-acid types selected.

pKAL Inhibitors

Inhibition of pKAL by EPI-K401 and EPI-K503. Since EPI-K401 expresses the consensus of Lib#4 selectants, we expressed this protein in *P. pastoris* to study its properties. It is a potent inhibitor of pKAL with $K_i = 284$ pM with $k_{on} = 0.21 \times 10^6$/M/s, giving $k_{off} = 56 \times 10^{-6}$/s. EPI-K401 has at least 100-fold higher affinity for pKAL than does aprotinin.

No isolate from Lib#5 exhibits the consensus, K5con. Two isolates differ by only one amino acid from K5con. Sequence K503 is close to the consensus and not as far from LACI-D1 or EPI-K401 as most other selectants. Thus, we chose to express EPI-K503 in *S. cerevisiae* to study its properties. EPI-K503 is a very potent inhibitor of pKAL, with $K_i = 40$ pM with $k_{on} = 1.5 \times 10^6$/M/s, giving $k_{off} = 61. \times 10^{-6}$/s. Hence, the improvement of EPI-K504 over EPI-K401 is in the on rate with a small loss in the off rate. There are thirty proteins that have either the K401 or the K503 residue at positions 13, 15, 17, 19, or 21. All of these intermediates were allowed, but none was observed following selection. EPI-K508 is the Lib#5 selectant closest to EPI-K401.

Specificity of EPI-K401 and EPI-K503. Both EPI-K401 and EPI-K503 are highly specific for pKAL. Of the proteases we tested, only PLA is inhibited to any great extent. EPI-K401 and EPI-K503 show similar degrees of discrimination between pKAL and PLA even though EPI-K503 shows 7-fold higher affinity for pKAL than does EPI-K401. The improvement in pKAL binding appears mostly in k_{on}; it is possible that EPI-K503 has structural features that favor accelerated binding in general.

For both EPI-K401 and EPI-K503, the level of specificity allows one to selectively inhibit pKAL in the presence of PLA. For example, if [PLA] = 1 nM and [pKAL] = 1 nM, then adding 4 nM of EPI-K503 would inhibit 99% of the pKAL and only 13% of the PLA. Obtaining 99% inhibition of 1 nM pKAL by EPI-K401 would require 28 nM inhibitor, which would inhibit about 22% of 1 nM PLA. Thus, both of these inhibitors can block essentially all pKAL activity while leaving PLA and other proteases substantially uninhibited.

Stability of EPI-K401. EPI-K401 retains the high degree of stability characteristic of Kunitz domain proteins. Although EPI-K401 is stable over a wide range of pH, it is not so pH stable as is BPTI (data not shown). EPI-K401 has stability similar to other LACI-D1 derivatives (*e.g.*, EPI-P302) that we have studied. The pKAL-inhibiting activity of EPI-K401 is highly resistant to oxidants even though the molecule contains two methionines (M_1 and M_{54}). We presume that these are oxidized at least to the sulfoxide, but this does not change the pKAL binding because they are far from the binding interface. EPI-K401 is slightly more heat stable and less pH stable than EPI-P302.

Determinants of pKAL Inhibition. Note that the changes between K401 and K503 are mostly conservative: W for F, A for N, R for K, and G for A. The alteration H13P is the least conservative. It is interesting that the residues that differ between K401 and K503 are 13, 15, 17, 19, and 21. This segment of canonical Kunitz domains forms a fairly flat curved band with the side groups being above or below the plane of the band. The side groups of 13, 15, 17, 19, and 21 lie on one side of the band while the residues 14, 16, 18, and 20 lie on the other side. It is possible that the even-numbered residues make contacts with pKAL that are very favorable to binding while the odd-numbered residues either contact residues that are less well localized (and so can taken on multiple conformations) or do not make direct contact with pKAL.

Possible Clinical Implications. Wachtfogel *et al*. (1995) report that specific inhibitors of pKAL can prevent release of HNE from neutrophils during simulated extracorporeal blood circulation. None of the inhibitors tested was nearly as potent in inhibiting pKAL as are EPI-K401 or EPI-K503. In particular, Wachtfogel *et al*. (1995) found that efficacy correlated best with high on rate. EPI-K503 has an on rate equal to that of Bz-Pro-Phe-boroArg-OH (the fastest inhibitor tested by Wachtfogel) with 2.7×10^5-fold slower off rate. Thus, EPI-K503 is likely to be more effective than any of

the pKAL inhibitors tested by Wachtfogel *et al*. (1995). The combination of small size, high affinity, specificity, and stability make EPI-K401 and EPI-P503 attractive candidates for development as therapeutic or imaging agents.

THBN

Lib#6 and THBN-Binding Lib#6 Selectants. Lib#6 differs from Lib#2 and Lib#4 only in that it was constructed from a population of Lib#1 that was selected for binding to THBN. Table 10 contains the sequences of 13 isolates from Lib#6 selected for binding to THBN through three rounds. No two clones had identical sequences. T612 is unusual in having C_{39}; this clone also carries the mutation C30G so that the number of cysteines is conserved. Possibly a novel set of disulfides presents the THBN-binding surface in a suitable way to allow selection of the clone. The degree of selection in the P1 region of T601−T612 is extraordinary; with the exception of T610, all the clones are identical for residues 13−19. The second-loop positions (31, 32, 34, and 39) are not strongly selected. At position 31, E is slightly preferred. At position 32, E is fairly well preferred (9/12). At position 34, no basic types are seen, but little other selection is seen. At position 39, more selectants have T than any other type, but N, R, K, G, L, and C also occur. Of the eleven isolates, eight have a hydrophilic or basic residue at position 39.

Le Bonniec *et al.* (1993) report that THBN($\Delta P_{60B}P_{60C}W_{60D}$) is inhibited by BPTI with $K_i = 16$ nM, indicating that there is space enough for a Kunitz domain to fit into the active site of THBN when these residues are not present. Presumably, these residues are displaced when THBN cleaves a protein substrate. We considered two ways in which a Kunitz domain could inhibit THBN: (1) push PPW aside and bind in a mode similar to BPTI binding trypsin and (2) binding to the "B-loop" in a novel geometry that nevertheless occludes the active site. The so-called "B-loop" projects from the body of thrombin (Bode *et al*., 1989), and it is likely that it changes conformation when thrombomodulin binds. Although the PPW residues may exclude BPTI when in the position observed for chloromethyl ketone-inhibited THBN, it is possible that the loop could be repositioned by an inhibitor that binds with sufficient affinity.

T601 and T602 were purified by binding to THBN-agarose. We have found with other proteins that such purification succeeds when there is substantial binding. Theoretically, there could be no purification if there were no binding. Because phage that display T601 or T602 do not bind to PLA-agarose, trypsin-agarose, or pKAL-agarose, we assume that the T601 and T602 domains do not bind agarose *per se*. Thus, T601 and T602 bind THBN or THBN-agarose. Since the THBN-selected proteins do not inhibit THBN, we assume that they do not bind the active site of THBN. As shown in Table 11, the binding of phage that display T601 or T602 to THBN-agarose is strong and highly specific.

Lib#1 and Lib#6 may not contain the right set of substituents to complement THBN well enough to displace PPW. If a Kunitz domain is to bind and inhibit THBN, then other Kunitz domain libraries might be needed. In any even, the site on THBN that binds the non-inhibitor motif $d_{10}d_{11}$-$gR_{13}ckGDGG_{19}$ selects these phage very efficiently and could prevent selection of inhibitors even if they are present in the population. It is possible that $d_{10}dgRckgdgg_{19}$ Kunitz domains bound near the active site of THBN in such a way that inhibitory domains could not bind, but it is more likely that this motif bound a distant site very efficiently and crowded out any true inhibitors that might have been in the population. If one wished to produce a Kunitz domain THBN inhibitor, then it would need to be selected in one of three (not mutually exclusive) ways: (a) from a library that lacks the $R_{13}ckGDGG_{19}$ motif and similar motifs, (b) from the presence of excess EPI-T601 or a similar molecule that would saturate sites (assuming the site is distant from the catalytic site) to which this motif binds [see Dennis and Lazarus (1994b)], or (c) from use of the THBN−thrombomodulin complex as target since this complex exhibits broader specificity than does THBN (indicating that the active site is more accessible).

This study shows that Kunitz domains can bind to proteins at sites other than the catalytic site of a trypsin-homologous serine protease through the P1 region. Secondly, one cannot assume that a protease-inhibitor backbone will home-in on the active site of a protease.

CONCLUSIONS

Starting from the same library as used for PLA, we used phage display iteratively to select better and better inhibitors of pKAL, generating small, stable nearly human Kunitz domain molecules with high affinity and specificity. It appears that variation of residues near the interface (as judged from the BPTI:trypsin model) plays an important role in conferring on Kunitz domains very high affinity for a particular protease. The resulting molecules are candidate therapeutics, candidate imaging agents, and candidate lead compounds for pharmaceutical drug development. Further iterations may improve the affinity even further. With the inhibitors EPI-P302 (against PLA) and EPI-K503 (against pKAL), one can discover which activity of BPTI is important in reducing perioperative bleeding.

It appears, from the improvement between EPI-K401 and EPI-K503, that customizing a Kunitz domain to have high affinity for a target (K_i < 100 pM) does not necessarily reduce the specificity relative to other proteases.

The molecules selected for THBN binding do not inhibit and are likely to bind at a site other than the catalytic site, demonstrating that Kunitz domains are not limited to the active site of trypsin-homologous proteases as binding sites.

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BI952629Y