# Kinetic Characterization of Two Active Mutants of Placental Ribonuclease Inhibitor That Lack Internal Repeats†

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ABSTRACT: Human placental ribonuclease inhibitor (PRI), a 50-kDa tight-binding inhibitor of angiogenin and pancreatic ribonuclease, consists predominantly of 7 internal repeats, each 57 residues long. Repeats 3 plus 4 (residues 144–257) or repeat 6 (residues 315–371) can be deleted to give mutant proteins, PRIΔ3–4 and PRIΔ6, respectively, that retain inhibitory activity [Lee, F. S., & Vallee, B. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1879–1883]. We describe here the isolation and characterization of these two active mutant proteins. Both inhibit the enzymatic activities of either angiogenin or bovine pancreatic ribonuclease A (RNase A) with a 1:1 stoichiometry, and the mode of inhibition of RNase A by either is competitive. PRIΔ3–4 binds to angiogenin and RNase A with $K_i$ values of 0.72 and 170 pM, respectively. The corresponding values for PRIΔ6 are 22 and 43 pM, respectively. Since recombinant PRI binds to angiogenin and RNase A with $K_i$ values of 0.29 and 68 fM, respectively, deletion of repeats 3 plus 4 weakens both interactions 2500-fold while deletion of repeat 6 weakens them 76 000- and 630-fold, respectively. Therefore, either the deletion of these repeats has altered the conformation of the angiogenin/RNase binding site in PRI or the deleted repeats contribute directly to the binding site, or both. In addition, the tighter binding to angiogenin versus RNase A seen with native PRI has been preserved in PRIΔ3–4 but has been almost completely abolished in PRIΔ6. Despite the substantial weakening of affinity observed for these mutant proteins, a critical contact with the active-site Lys-40 of angiogenin that is present in the angiogenin·native PRI complex is also present in both mutant complexes: like native PRI, PRIΔ3–4 and PRIΔ6 bind substantially more weakly, 9200- and 6800-fold, respectively, to an angiogenin mutant in which Lys-40 has been changed to Gln than to native angiogenin.

Human placental ribonuclease inhibitor (PRI)[1] is a member of a family of acidic cytoplasmic proteins that occur in a wide variety of mammalian tissues (Blackburn et al., 1977; Blackburn & Moore, 1982). These proteins form extremely tight 1:1 complexes ($K_i \sim 10^{-15}$ M) (Lee et al., 1989a,b) with extracellular and intracellular ribonucleases and may play a role in controlling cytoplasmic RNA levels (Blackburn & Moore, 1982). PRI binds to angiogenin, a blood vessel inducing protein homologous to pancreatic ribonuclease (Fett et al., 1985; Strydom et al., 1985), and thereby abolishes both its angiogenic and its ribonucleolytic activities (Shapiro & Vallee, 1987). PRI binds to angiogenin even more tightly than to RNase A and may therefore also function in the regulation of angiogenin (Shapiro & Vallee, 1987; Lee et al., 1989a,b).

The primary structures of PRI and related inhibitors from HeLa cells and porcine liver all consist of seven tandem leucine-rich repeats flanked by shorter N- and C-terminal segments (Lee et al., 1988; Schneider et al., 1988; Hofsteenge et al., 1988). These repeats are each 57 residues long and ~40% identical with one another. Similar leucine-rich repeats have been observed in other proteins of diverse function (Lee et al., 1988; Schneider et al., 1988; Hofsteenge et al., 1988).

Deletion of repeats 3 plus 4 (residues 144–257) or repeat 6 (residues 315–371) of PRI results in two mutant proteins, PRIΔ3–4 and PRIΔ6, respectively, that inhibit RNase A (Lee & Vallee, 1990). Eighteen other deletions, including other single- and multiple-repeat deletions, all abolish this activity. Both for purposes of understanding the high affinity of the interaction and for providing a basis for rational inhibitor design, it is important to assess the tightness of binding of these inhibitors to angiogenin and RNase. We here describe the purification and characterization of these PRI mutant proteins. Deletion of repeats 3 plus 4 or repeat 6 of PRI weakens its interaction with angiogenin/RNase A 630–76 000-fold, but a critical contact with Lys-40 of angiogenin is still present in both angiogenin·mutant PRI complexes.

## Experimental Procedures

*Materials.* Oligonucleotides were synthesized on a Biotix Model 102 DNA synthesizer. Angiogenin for binding studies or enzymatic assay was obtained from mammalian or bacterial expression systems, respectively (Kurachi et al., 1988; Shapiro et al., 1988). The angiogenin mutant in which Lys-40 was changed to Gln by site-specific mutagenesis (K40Q angioge-

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[1] Abbreviations: PRI, placental ribonuclease inhibitor; PRIΔ3–4, PRI des-(144–257); PRIΔ6, PRI des-(315–371); RNase A, bovine pancreatic ribonuclease A; K40Q angiogenin, angiogenin mutant in which Lys-40 has been changed to Gln; HSA, human serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; C>p, cytidine cyclic 2′,3′-phosphate; CpG, cytidylyl(3′–5′)-guanosine; CpA, cytidylyl(3′–5′)adenosine; UpG, uridylyl(3′–5′)-guanosine; HPLC, high-performance liquid chromatography; NaDodSO$_4$–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; kb, kilobase(s); *n*, number of determinations.

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nin) was prepared as described (Shapiro et al., 1989; Lee & Vallee, 1989a). RNase A–Sepharose was prepared by the method of Blackburn (1979). The sources of all other materials have been described (Lee et al., 1988, 1989a,b; Shapiro et al., 1988). All buffers were degassed before use.

*Recombinant Plasmid Construction.* The plasmids pTRP-PRIΔ3–4 and pTRP-PRIΔ6 were derived from pTRP-PRI, which contains the *Escherichia coli trp* promoter and translation initiation sequence, the entire PRI coding sequence, and the 3′ untranslated region of the PRI cDNA (Lee & Vallee, 1989b). The plasmid pTRP-PRIΔ3–4 was constructed by first digesting pTRP-PRI with *Bst*XI and *Ppu*MI to remove the coding sequences for amino acids 1–258. The vector was then ligated with the 0.39-kb *Bst*XI/*Pvu*II restriction enzyme fragment of pTRP-PRI that encodes amino acids 1–130 and a synthetic duplex, comprised of oligonucleotides of the sequence CTGCTCTGCGAAGGACTCCTGGACCCCC-AGTGCCGCCTGAG and GTCCTCAGGCGGCACT-GGGGGTCCAGGAGTCCTTCGCAGAGCAG, that encodes amino acids 131 to 143 and 258.

The plasmid pTRP-PRIΔ6 was constructed in two steps. First, the 1.44-kb *Bst*XI/*Apa*I restriction enzyme fragment of pTRP-PRI that contains the entire PRI coding sequence was replaced with the 0.32-kb *Bsp*1286I restriction enzyme fragment of pTRP-PRI that encodes amino acids 374–460 and a synthetic duplex, comprised of oligonucleotides of the sequence GGTGCCCGACTGCTGTGTGAGACCCTGC-TGGAACCTGGCTGCCAGCTGCGGGTGCT and CCCGCAGCTGGCAGCCAGGTTCCAGCAGGGTC-TCACACAGCAGTCGGGCACCGCTC, that encodes amino acids 299–314 and 372–373. This duplex contains a *Ban*I site at amino acid 299. In the second step, the 1.56-kb *Bst*XI/*Eco*RI restriction enzyme fragment of pTRP-PRI that contains the entire PRI coding sequence was replaced with the 0.90-kb *Bst*XI/*Ban*I restriction enzyme fragment of pTRP-PRI that encodes amino acids 1–298 and the 0.49-kb *Ban*I/*Eco*RI restriction enzyme fragment of the product from the first step that encodes amino acids 299–314 and 372–460.

The identities of the recombinant plasmids were confirmed by restriction enzyme mapping and sequencing of the regions in which the synthetic oligonucleotide duplexes had been incorporated (Sanger et al., 1977; Chen & Seeburg, 1985).

*Expression and Isolation of PRI Mutant Proteins.* PRIΔ3–4 and PRIΔ6 were purified from *E. coli* strain W3110 transformed with pTRP-PRIΔ3–4 or pTRP-PRIΔ6, respectively, using a modification of the procedure employed for the isolation of native PRI from *Escherichia coli* (Lee & Vallee, 1989b). All steps were performed at 0–4 °C. Preparation of the bacterial extract up to and including the streptomycin sulfate precipitation step was identical with that previously described except that the bacterial pellet was resuspended in 32 mL of 50 mM Hepes, pH 7, containing 0.1 M NaCl, 1 mM EDTA, 10 mM DTT, and 1 mM phenylmethanesulfonyl fluoride.

The supernatant from the streptomycin sulfate precipitation was slowly adjusted to 25% $(NH_4)_2SO_4$ saturation, stirred an additional 40 min, and centrifuged at 17300*g* for 30 min. The supernatant was slowly adjusted to 55% $(NH_4)_2SO_4$ saturation and stirred and centrifuged as above. The pellet (~4 g) was dissolved in 10 mL of 50 mM Hepes, pH 7, containing 0.1 M NaCl, 1 mM EDTA, 5 mM DTT, and 15% glycerol (v/v) (buffer A) and dialyzed against two changes of 35 volumes of the same buffer over 12 h.

The solution was centrifuged at 50000*g* for 1 h, and the supernatant was applied to 2 mL of RNase A–Sepharose equilibrated with buffer A and packed in a 1 cm (i.d.) × 5 cm column. Chromatography was performed at a flow rate of 0.1 mL/min. The column was washed with 8–10 column volumes of buffer A supplemented with an additional 0.4 M NaCl and eluted as described (Blackburn, 1979). Fractions containing inhibitory activity toward RNase A were dialyzed against 20 mM Hepes, pH 7, containing 50 mM NaCl, 1 mM EDTA, 5 mM DTT, and 15% glycerol (v/v) in a Centricon 30 microconcentrator (Amicon) and stored at 4 °C. No loss of activity for either mutant was observed when stored under these conditions for at least 2 months.

*Protein Concentration Measurements.* The concentrations of angiogenin and RNase A stock solutions were determined as described (Lee et al., 1989a). For specific activity and inhibition stoichiometry measurements, PRI mutant protein concentrations were estimated from $A_{280}$ by using molar absorptivities based on their expected amino acid compositions (Wetlaufer, 1962). One unit of inhibitor activity equals that amount necessary to inhibit 5 ng of RNase A by 50% (Shortman, 1961). For kinetic analyses, mutant protein concentrations were determined by inhibition of RNase A activity toward C>p (Blackburn, 1979) with a titration plot consisting of three points. These plots are linear under the conditions employed; i.e., [PRI] $\gg K_i$ for the inhibition of RNase A by either of the two PRI mutants. PRI mutant protein concentrations determined by the two methods generally differed by no more than 5%.

*Enzyme Assays.* (*A*) *Specific Activity and Inhibition Stoichiometry Measurements.* Assays measuring inhibition of RNase A or angiogenin enzymatic activity employed the substrates C>p or CpG, respectively, and cleavage was monitored by means of a spectrophotometer or an HPLC system, respectively, as described (Lee et al., 1989a; Shapiro et al., 1986, 1988). The enzyme and substrate concentrations for RNase A assays were 73 nM and 1 mM, respectively. Those for angiogenin assays were 1 and 100 μM, respectively, and incubations for these assays were performed at 22 °C.

(*B*) *Henderson Plots.* Assays measuring the inhibiton of RNase A by the PRI mutants employed the dinucleotide CpA as substrate (Witzel & Barnard, 1962) and were performed essentially as described (Lee et al., 1989b). The mutant protein concentrations ranged from 0.2 to 0.8 nM for PRIΔ6 and from 0.6 to 2.4 nM for PRIΔ3–4. RNase A was preincubated with the PRI mutant for 10 min before addition of substrate. Substrate cleavage was monitored at wavelengths from 286 to 298 nm, depending on the substrate concentration.

(*C*) *Competition Experiments.* PRI mutant protein was added to a mixture of 1 equiv of RNase A and either 0.6 to 1.8 equiv of angiogenin or 6 to 300 equiv of K40Q angiogenin at 25 °C. After 30 min, 12 μL of substrate, UpG, was added to give a final concentration of 100 μM in 600 μL. Buffer was 0.1 M Mes, pH 6, containing 0.1 M NaCl, 1 mM EDTA, 100 μM DTT, and 20 μg/mL HSA. The final total RNase A concentration was 15 nM. The activity of free RNase A was determined spectrophotometrically as described (Lee et al., 1989a). In control assays, both PRI mutant protein and either angiogenin or K40Q angiogenin was omitted.

*Angiogenin·PRIΔ3–4 Kinetics.* The association and dissociation rate constants for the angiogenin·PRIΔ3–4 interaction were determined as described (Lee et al., 1989ab). In measurement of the dissociation rate, the final concentration of angiogenin was 0.70 μM.

*Physicochemical and Immunological Analyses.* NaDodSO$_4$–PAGE was performed as described (Laemmli, 1970). Molecular weight markers were obtained from Pharmacia

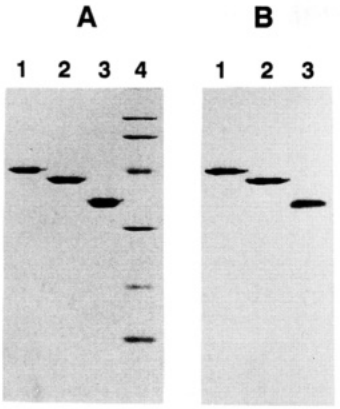


FIGURE 1: $NaDodSO_4$–PAGE (A) and immunoblot (B) analysis of native PRI (lane 1), PRIΔ6 (lane 2), and PRIΔ3–4 (lane 3). The amount of PRI or PRI mutant protein loaded per lane was either 2 (A) or 0.5 μg (B). The sizes of molecular weight standards [(A) lane 4], from top to bottom, are 94K, 67K, 43K, 30K, 20K, and 14K. $NaDodSO_4$–PAGE analysis was conducted using a 12.5% separating gel with a 5% stacking gel. Proteins were visualized by using either Coomassie Blue R-250 (A) or affinity-purified, polyclonal, anti-PRI antibodies (B) followed by treatment as described (Kurachi et al., 1988).

LKB Biotechnology. Immunoblotting was conducted with affinity-purified, polyclonal, anti-PRI antibodies prepared as described (Lee et al., 1988). Amino acid analysis was performed by the Pico-Tag method (Bidlingmeyer et al., 1984). Prior to analysis, PRI mutant proteins were dialyzed into water in Centricon 30 microconcentrators.

## RESULTS

*Isolation of PRI Mutant Proteins.* The yields of the two PRI mutants from *E. coli* by the usual affinity chromatography on RNase A–Sepharose (Lee & Vallee, 1989b) were low, 130 μg of PRIΔ3–4 and none of PRIΔ6 from 4 L of bacterial culture. The purification procedure was therefore modified. First, an ammonium sulfate precipitation step was introduced before the affinity chromatography step. Second, the loading buffer for affinity chromatography was changed from a pH 6.4, phosphate, to a pH 7, Hepes buffer, and the flow rate employed was reduced from 38 to 8 cm/h. These modifications improved the recoveries of mutant proteins from the affinity chromatography step and increased the yields of PRIΔ3–4 and PRIΔ6 to 760 and 170 μg, respectively, in greater than 95% purity (Figure 1A). The specific activities of PRIΔ3–4 and PRIΔ6 were 147 000 and 125 000 units/mg, respectively, essentially the values expected for full specific activity.

*Physicochemical and Immunological Characterization.* The differences in apparent molecular weights between native PRI[2] and either PRIΔ6 or PRIΔ3–4 are close to those expected from the size of the deletions made, i.e., one repeat unit in PRIΔ6 and two in PRIΔ3–4 (Figure 1A). The molecular weight values calculated by comparison with molecular weight standards are 40 000 for PRIΔ6 and 35 000 for PRIΔ3–4 and are 3000–4000 lower than expected for each mutant protein. However, native PRI itself, a 50-kDa protein, migrates with an apparently low molecular weight of 43 000 in this particular $NaDodSO_4$–PAGE system. Both mutant proteins, like native PRI, are immunoreactive toward anti-PRI antibodies (Figure 1B).

Table I: Amino Acid Compositions of PRI Mutant Proteins[a]

| amino acid | residues/mol PRIΔ6 | residues/mol PRIΔ3–4 |
|---|---|---|
| Asx | 39.8 (40) | 32.6 (33) |
| Glx | 49.3 (51) | 44.9 (47) |
| Ser | 32.0 (37) | 27.8 (32) |
| Gly | 29.1 (28) | 24.6 (24) |
| His | 4.3 (4) | 4.2 (4) |
| Arg | 21.4 (21) | 19.4 (19) |
| Thr | 12.3 (12) | 11.3 (11) |
| Ala | 29.6 (28) | 23.4 (22) |
| Pro | 13.6 (13) | 9.5 (9) |
| Tyr | 2.9 (3) | 2.0 (2) |
| Val | 19.8 (21) | 17.2 (18) |
| Met | 1.1 (2) | 0.7 (1) |
| Ile | 10.4 (11) | 9.8 (10) |
| Leu | 86.0 (83) | 75.2 (72) |
| Phe | 1.2 (1) | 3.2 (3) |
| Lys | 16.1 (15) | 9.8 (9) |
| Cys | 27.7 (28) | 25.0 (24) |
| Trp | 4.8 (5) | 5.6 (6) |

[a] Pico-Tag method (Bidlingmeyer et al., 1984). Cys determined as cysteic acid after performic acid oxidation. Trp determined after hydrolysis with methanesulfonic acid. Average of duplicate analyses. Number of residues expected based on the cDNA sequence and the specific deletions made are given in parentheses and exclude the initiator Met.

The amino acid compositions of both PRI mutant proteins are in good agreement with those expected (Table I). Like native PRI, the Leu, Cys, Asx, and Glx content of both mutant proteins is high. Further, the amino acid composition (Table I) and near-UV absorption spectra (not shown) reflect the relatively high Trp:Tyr ratios of the two mutant proteins.

*Stoichiometry of Inhibition.* A 1.0–1.1 equiv sample of each mutant essentially abolishes the enzymatic activity of either angiogenin or RNase A, consistent with a 1:1 inhibition stoichiometry. *p*-(Hydroxymercuri)benzoate (1 mM in the assay), which inactivates native PRI (Blackburn et al., 1977), completely reverses inhibition of RNase A by either mutant.

*Determination of $K_i$ Values.* Because the dissociation rate constants of the native PRI complexes with both RNase A and angiogenin are extremely low ($t_{1/2}$ ~0.5 day to months), the $K_i$ values for these interactions were calculated from the association and dissociation rate constants (Lee et al., 1989ab). The dissociation of each PRI mutant from its complex with either RNase A or angiogenin was examined by the same methods. In contrast to the RNase A·native PRI complex, the two RNase A·mutant PRI complexes both dissociate sufficiently rapidly ($t_{1/2}$ ~0.5–1.0 min) so that inhibition can be examined by steady-state kinetic methods. The dissociation of the angiogenin·PRIΔ6 complex is also relatively fast ($t_{1/2}$ ~2–4 min), but due to limitations of the nature of the assays for the ribonucleolytic activity of angiogenin, inhibition cannot be examined in an analogous manner. The $K_i$ value for PRIΔ6 inhibition of angiogenin was therefore obtained by examining the partitioning of inhibitor between angiogenin and RNase A. For the angiogenin·PRIΔ3–4 complex, the dissociation rate is sufficiently slow (see below) that the $K_i$ value must be calculated from the association and dissociation rate constants.

*RNase A Inhibition.* RNase A inhibition by both PRI mutants was examined by means of Henderson plots (Figure 2), which take into account inhibitor depletion (Henderson, 1972) and are appropriate when the measured initial velocities reflect steady-state conditions. To satisfy this condition, the enzyme concentration and substrate were chosen so that initial velocities could be measured over a time period that is greater

[2] For simplicity, we will refer to full-length PRI expressed in *E. coli* as native PRI. Recombinant PRI lacks the blocked N-terminus of PRI isolated from human placenta but is otherwise indistinguishable from the latter in its physicochemical, immunological, and kinetic characteristics (Lee & Vallee, 1989b).

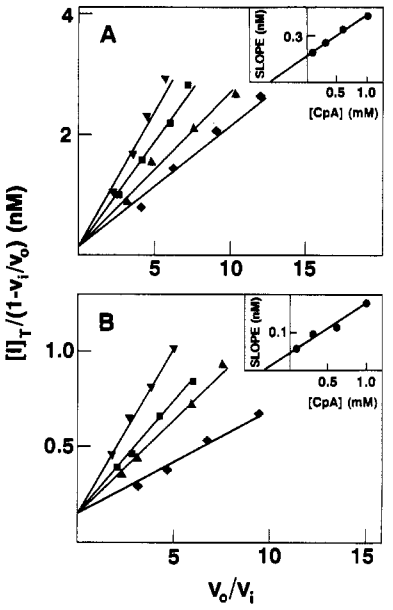


FIGURE 2: Henderson plots for inhibition of RNase A by PRIΔ3–4 (A) or PRIΔ6 (B). RNase A (150 pM) was preincubated with PRI mutant for 10 min at 25 °C followed by addition of CpA to final concentrations of 100 (♦), 300 (▲), 600 (■), or 1000 (▼) μM. Buffer was 0.1 M Mes, pH 6, containing 0.1 M NaCl, 1 mM EDTA, 50 μM DTT, and 20 μg/mL HSA. $[I]_T$ is the total PRI concentration, and $v_i$ and $v_o$ are the initial velocities in the presence and absence of inhibitor, respectively. Insets: Replot of slopes of Henderson plots versus substrate concentration. The line is obtained from linear regression of the data by using a weighting factor of $(1/\text{slope})^2$.

than 10 half-lives of the dissociation for either RNase A·inhibitor complex; during this time, less than 10% of the substrate was cleaved.

The slopes of the Henderson plots for both PRIΔ3–4 and PRIΔ6 increase linearly with increasing substrate concentration (Figure 2, insets), consistent with a competitive mode of inhibition. The absolute values of the $y$ and $x$ intercepts should equal the $K_i$ and $K_m$ values for inhibitor and substrate, respectively. The $K_i$ values for RNase A inhibition by PRIΔ3–4 and PRIΔ6 are 170 and 43 pM, respectively. From the Henderson plots for PRIΔ3–4 or PRIΔ6 inhibition, the $K_m$ value for CpA is either 0.62 or 0.32 mM, respectively; these values are in agreement with that of 0.32 mM reported previously (Witzel, 1963).

*Angiogenin Inhibition.* Tryptophan fluorescence is enhanced 50% in the angiogenin·native PRI complex and can serve to monitor the association of the two proteins by stopped-flow spectrophotometry (Lee et al., 1989a). Tryptophan fluorescence of the angiogenin complex with PRIΔ3–4 is enhanced but not that of its complex with PRIΔ6, which is slightly quenched (data not shown). The fluorescence change of the angiogenin·PRIΔ3–4 complex but not that of the angiogenin·PRIΔ6 complex is sufficiently large to monitor the association of the the two proteins.

For the angiogenin·PRIΔ3–4 interaction, the dependence of the pseudo-first-order rate constant of association on angiogenin concentration (Figure 3) is consistent with the two-step mechanism previously described for native PRI and angiogenin (Lee et al., 1989a). In this mechanism, the first step, a rapid equilibrium resulting in the formation of an enzyme·inhibitor complex, is followed by the second, a slower isomerization to a tighter complex. From Figure 3, the values for $K_1$, the dissociation constant of the first step, $k_2$, the rate constant of the second step, and $k_2/K_1$, the apparent second-order rate constant of association, are 0.82 μM, 95 $s^{-1}$, and $1.2 \times 10^8$ $M^{-1}$ $s^{-1}$, respectively.

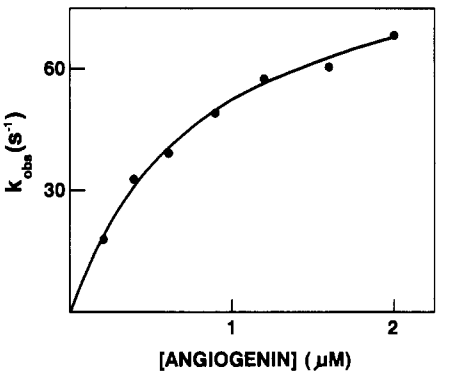


FIGURE 3: Association kinetics of PRIΔ3–4 with angiogenin. Shown is the dependence of the pseudo-first-order rate constant, $k_{obs}$, on angiogenin concentration. Tryptophan fluorescence was monitored with a 340-nm band-pass filter. Excitation was at 285 nm. Buffer was 0.1 M Mes, pH 6, containing 0.1 M NaCl and 1 mM EDTA at 25 °C. The curved line is calculated from eq 1 of Lee et al. (1989a) and the parameters $K_1 = 0.82$ μM and $k_2 = 95$ $s^{-1}$ obtained by linear regression of a double-reciprocal plot of the data using a weighting factor of $k_{obs}^2$. The value of $k_{-2}$ in eq 1 is extremely low (Figure 4) and can be neglected for these calculations.

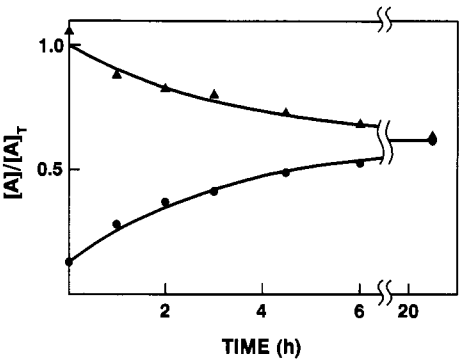


FIGURE 4: Dissociation kinetics of the angiogenin·PRIΔ3–4 complex. Angiogenin was preincubated with 1.5 equiv of PRIΔ3–4 for 20 min at 25 °C. Release of free angiogenin, A, was determined by HPLC after addition of 250 equiv of RNase A (●). A second sample contained identical components except that angiogenin was added last (▲). $[A]_T$ is the total angiogenin concentration. The buffer was 0.1 M Mes, pH 6, containing 0.1 M NaCl, 1 mM EDTA, and 300 μM DTT. The line showing release of free angiogenin is calculated from the equation $[A] = [A]_{eq} - ([A]_{eq} - [A]_o)e^{-k_d t}$, where $[A]_{eq}$ is the free angiogenin concentration at equilibrium, $[A]_o$ is that observed at the first time point after addition of scavenger, and $k_d$ is the dissociation rate constant of the angiogenin·PRIΔ3–4 complex. The value of $k_d$, $8.3 \times 10^{-5}$ $s^{-1}$, is from a plot of $-\ln\{([A]_{eq} - [A])/([A]_{eq} - [A]_o)\}$ vs $t$.

The dissociation rate constant of the angiogenin·PRIΔ3–4 complex was determined by monitoring the release of free angiogenin from the complex as a function of time in the presence of 250 equiv of RNase A as scavenger for free PRI (Lee et al., 1989b). In the absence of scavenger, free angiogenin is not released from the complex. In its presence, a small amount of free angiogenin, 13% of the total, became available within several minutes after addition of scavenger while the remainder was released more slowly (Figure 4). The dissociation rate constant was calculated from the slow phase.

After 20.5 h, an apparent equilibrium is reached where only 62% of the total angiogenin is free. Another sample in which angiogenin was added last to a mixture of RNase A and PRIΔ3–4 also reaches the same free angiogenin concentration (Figure 4). The apparent equilibrium is not due to a decrease in total angiogenin, since a control sample lacking inhibitor shows no loss of angiogenin over the time course of the experiment. If the apparent equilibrium at 20.5 h is a true equilibrium, the calculated dissociation rate of the complex

is $8.3 \times 10^{-5}$ s$^{-1}$ ($t_{1/2}$ = 2.3 h). The $K_i$ value for angiogenin inhibition by PRIΔ3–4, 0.72 pM, is calculated from the association and dissociation rate constants by the equation $K_i = K_1 k_{-2}/(k_{-2} + k_2)$.

The rapid dissociation of the angiogenin·PRIΔ6 complex precludes an analogous determination of the dissociation rate constant and $K_i$ value. The $K_i$ value for angiogenin inhibition by PRIΔ6 was therefore determined by examining the competition between angiogenin and RNase A for inhibitor at equilibrium. PRIΔ6 was added to a mixture of angiogenin and RNase A, and after equilibration, the partitioning of inhibitor between angiogenin and RNase A was determined by assay of free RNase A. The incubation time was 30 min, which is more than seven half-lives of dissociation of either of the two complexes in the mixture. In addition, incubation of PRI mutant with either angiogenin or RNase A before addition of the other enzyme and followed by the same 30-min incubation resulted in the same activity. The $K_i$ value for angiogenin inhibition by PRIΔ6, $K_{i,A}$, can be calculated by the equation:

$$\alpha = K_{i,A}/K_{i,R} = [A]_F[R{\cdot}I]/[R]_F[A{\cdot}I] \quad (1)$$

where $K_{i,R}$ is the PRIΔ6 inhibition constant for RNase A, F indicates free concentration at equilibrium, and A, R, and I denote angiogenin, RNase A, and inhibitor, respectively.

Experimentally, the free RNase A concentration, $[R]_F$, is determined by the activity toward UpG, which is not cleaved appreciably by angiogenin under the assay conditions (Shapiro et al., 1986), at a substrate concentration, 100 μM, that is well below the $K_m$, 2.0 mM, reported for comparable conditions (Follman et al., 1967). Concentrations of all other components are calculated from $[R]_F$ by assuming that any inhibitor not bound to RNase A is bound to angiogenin since the inhibitor concentration employed, 15 nM, is 350-fold higher than its $K_i$ value for RNase A inhibition. The experimental value of $\alpha$, 0.51 ± 0.16 ($n$ = 6), combined with the $K_{i,R}$ value of 43 pM obtained from Henderson plots yields a $K_i$ value of 22 pM for angiogenin inhibition by PRIΔ6.

*K40Q Angiogenin Binding.* The interactions of either PRIΔ3–4 or PRIΔ6 with an angiogenin mutant in which the active site lysine, Lys-40, has been changed to Gln (K40Q angiogenin) were examined by the same equilibrium competition experiments just described for PRIΔ6 and angiogenin. The dissociation of K40Q angiogenin from its complexes with either PRIΔ3–4 or PRIΔ6 is faster than that observed for native angiogenin and PRIΔ6 (data not shown). Therefore, the incubation time previously employed, 30 min, should allow the attainment of equilibria in these experiments. The values of $\alpha$ and $K_i$ obtained for K40Q angiogenin binding to PRIΔ3–4 are 39 ± 7 ($n$ = 6) and 6.6 nM, respectively. The corresponding values for the K40Q angiogenin·PRIΔ6 interaction are 3500 ± 600 ($n$ = 4) and 150 nM, respectively.

## Discussion

Detailed kinetic examination of active PRI mutant proteins that lack one or more internal repeats may yield important information about the angiogenin/RNase A binding site of PRI. Such information should enlarge the understanding of the high affinity of the interaction and provide a basis for rational inhibitor design. Therefore, two active PRI mutant proteins identified previously (Lee & Vallee, 1990) were isolated and characterized.

We show here that deletion of repeats 3 plus 4 of PRI results in a mutant protein that binds to angiogenin and RNase A with $K_i$ values of 0.72 and 170 pM, respectively (Table II); the corresponding values for the deletion of repeat 6 are 22 and 43 pM, respectively. Since native PRI expressed in *E. coli* binds to angiogenin and RNase A with $K_i$ values of 0.29 and 68 fM, respectively (Lee & Vallee, 1989b), deletion of repeat 6 weakens these interactions 76 000- and 630-fold, respectively, whereas deletion of repeats 3 plus 4 weakens them both 2500-fold. Therefore, either the deletion of these repeats has altered the conformation of the angiogenin/RNase binding site in PRI or the deleted repeats contribute directly to the binding site, or both.[3]

Table II: Inhibition Constants for PRI, PRIΔ3–4, and PRIΔ6[a]

| inhibitor | $K_i$ (pM) RNase A | angiogenin | K40Q | $K_{i,R}/K_{i,A}$ | $K_{i,Q}/K_{i,A}$ |
|---|---|---|---|---|---|
| PRI | 0.068[b] | 0.00029[b] | 0.93[c] | 230 | 3200 |
| PRIΔ3–4 | 170 | 0.72 | 6600 | 240 | 9200 |
| PRIΔ6 | 43 | 22 | 150000 | 2 | 6800 |

[a] Conditions are 0.1 M Mes, pH 6, 0.1 M NaCl, 1 mM EDTA, and 25 °C. $K_{i,R}$, $K_{i,A}$, and $K_{i,Q}$ denote inhibition constants for RNase A, angiogenin, and K40Q angiogenin, respectively. [b] Values are for native PRI expressed in *E. coli* and are from Lee and Vallee (1989b). [c] Value is for native PRI isolated from human placenta and is from Lee and Vallee (1989a).

A PRI repeat unit could contribute to the binding site if it made direct contact with the angiogenin/RNase molecule. A critical contact identified previously involves the active-site Lys-40 of angiogenin; mutagenesis of Lys-40 to Gln weakens the binding to native PRI by ~3 orders of magnitude (Lee & Vallee, 1989a). Conceivably, a substantial decrease in affinity of the PRI mutants for angiogenin could be due to the loss of this single contact alone. To examine this possibility, the affinity of both PRI mutants for the K40Q angiogenin mutant was measured. If the critical contact with Lys-40 of angiogenin is absent, then the PRI mutants should show comparable affinities for the mutant and native angiogenins; if it is present, then the binding of the PRI mutants to the mutant angiogenin should be substantially weaker than that to native angiogenin.

PRIΔ3–4 and PRIΔ6 bind 9200- and 6800-fold more weakly, respectively, to K40Q angiogenin than to native angiogenin (Table II). The differences in affinities are comparable to that observed for native PRI and strongly suggest that the critical contact with Lys-40 of angiogenin has been preserved in both PRI mutant complexes. Hence, the chemical moiety in PRI that contacts Lys-40, likely an acidic one (Lee & Vallee, 1989a), is present in both mutants and is not located in repeat units 3, 4, or 6. Preservation of this contact with Lys-40 is consistent with the finding that both PRIΔ3–4 and PRIΔ6, like native PRI, are competitive inhibitors of RNase A (Figure 2).

It may also be noted that the $K_i$ values for inhibition of either angiogenin or RNase A by both mutant proteins are in all cases subnanomolar, indicating that these mutants are still potent inhibitors of the two enzymes. Thus, despite weakening affinity substantially, the deletions examined have not altered the angiogenin/RNase binding site catastrophically.

Nevertheless, the changes in $K_i$ value indicate some degree of perturbation of the angiogenin/RNase binding site. The deletions may have removed portions of the contact region or

[3] Since PRI has an internal repeat structure, it could be argued that the primary binding site is absent in both mutant proteins and that the binding observed is that of a second, weaker, binding site. This is unlikely, however, since a 1:1 stoichiometry of inhibition of either angiogenin or RNase A by native PRI is measured at enzyme concentrations ≫1 nM (Lee & Vallee, 1989b), conditions under which a second binding site with a subnanomolar $K_i$ value (Table II) would be expected to yield an enzyme/inhibitor stoichiometry of 2:1.

Table III: Kinetic Parameters for Inhibition of Angiogenin by PRI and PRIΔ3-4[a]

| inhibitor | $K_1$ (μM) | $k_2$ ($s^{-1}$) | $k_2/K_1 \times 10^{-8}$ ($M^{-1}$ $s^{-1}$) | $k_{-2} \times 10^7$ ($s^{-1}$) | $K_i$ (fM) |
|---|---|---|---|---|---|
| PRI[b] | 0.67 | 120 | 1.8 | 0.52 | 0.29 |
| PRIΔ3-4 | 0.82 | 95 | 1.2 | 830 | 720 |

[a] Conditions are 0.1 M Mes, pH 6, 0.1 M NaCl, 1 mM EDTA, and 25 °C. $K_1$, $k_2$, and $k_{-2}$ refer to the two-step mechanism

$$E + I \overset{K_1\ (fast)}{\rightleftharpoons} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^*$$

where E is enzyme, I is inhibitor, and the asterisk denotes a tighter form of the EI complex (Lee et al., 1989a). [b] Values are for native PRI expressed in *E. coli* and are from Lee and Vallee (1989b).

indirectly altered the conformation of the binding site. One possibility is that the topology of the binding site may be established from regions distant in the primary structure and be altered due to deletion of intervening repeats. Another possibility, not mutually exclusive, is that the juxtaposition of repeats separated in the primary structure could account for conformational changes near or distant to that site.

Data for the association of PRIΔ3-4 with angiogenin (Figure 3) are consistent with the same two-step mechanism which was observed for native PRI and angiogenin (Lee et al., 1989a); the values for the association rate constants are, in fact, comparable to those for native PRI and angiogenin (Table III). Most of the increase in the $K_i$ value for PRIΔ3-4 is due to a 1600-fold increase in the dissociation rate constant (Table III). An independent estimate of the $K_i$ value can be made by using eq 1 to examine the apparent equilibrium attained in the experiment that measures the dissociation rate of the complex (Figure 4). By determining all enzyme and enzyme·inhibitor concentrations from the amount of free angiogenin measured at the apparent equilibrium (at 20.5 h) and with the value of 170 pM obtained for $K_{i,R}$ by Henderson plots, a value of 1.2 pM for $K_{i,A}$ is obtained, which is in reasonable agreement with that calculated, 0.72 pM.

PRIΔ3-4 binds 240-fold more tightly to angiogenin than to RNase A, similar to the situation with native PRI (Table II). PRIΔ6, however, only binds 2-fold more tightly. Therefore, the binding of native PRI to angiogenin tighter than that to RNase A is not solely due to a difference in bulk properties of the two enzymes, e.g., differences in basicity (Lee et al., 1989b). Properties of the binding site of native PRI itself which appear to have been altered in PRIΔ6 must contribute to the difference in affinity, e.g., the capacity to form specific salt bridges, hydrogen bonds, and/or van der Waals contacts. In principle, it should be possible to obtain additional PRI derivatives whose enzyme specificities are altered.

Why should native PRI have to contain seven internal repeats when these results suggest that a lesser number would suffice to bring about inhibition? On the basis of the lower $K_i$ values observed for native PRI (approximately femtomolar) compared to those of its mutants (approximately picomolar to nanomolar), one might speculate that the full complement of repeats is necessary for binding to be tight enough for the physiological functions of PRI. However, it is also conceivable that other deletion mutants might be equally as potent as the native protein or that the repeats which are nonessential for inhibition may serve as yet unknown functions. In this regard, future studies should clarify the role of the internal repeats in PRI, define more specifically the angiogenin/RNase A binding site in PRI, and provide additional information for the rational design of novel antiangiogenic agents.

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