# Binding of Placental Ribonuclease Inhibitor to the Active Site of Angiogenin†

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ABSTRACT: The importance of specific residues in angiogenin for binding to placental ribonuclease inhibitor (PRI) has been assessed by examining the interaction of angiogenin derivatives with PRI. PRI binds native angiogenin with a $K_i$ value of $7.1 \times 10^{-16}$ M [Lee, F. S., Shapiro, R., & Vallee, B. L. (1989) *Biochemistry 28*, 225–230]. Substitution of a Gln for Lys-40 in angiogenin by site-specific mutagenesis decreases the association rate constant 3-fold and increases the dissociation rate constant 440-fold, resulting in a 1300-fold weaker $K_i$ value. The half-life of the mutant·PRI complex is 3.4 h compared to ~60 days for the native angiogenin·PRI complex. The magnitude of the change in $K_i$ value suggests that in the complex, Lys-40 forms a salt bridge or hydrogen bond with an anionic moiety in PRI. Carboxymethylation of His-13 or His-114 with bromoacetate increases the $K_i$ value 15-fold, and oxidation of Trp-89 by means of dimethyl sulfoxide and hydrochloric acid increases it 2.4-fold, suggesting that these residues also form part of the contact region with PRI. The changes in $K_i$ value reflect an increase in the dissociation rate constant. On the other hand, dinitrophenylation of either Lys-50 or Lys-60 with 1-fluoro-2,4-dinitrobenzene does not significantly alter the $K_i$ value, suggesting that these residues are not part of the contact region. These results indicate that PRI inhibition minimally involves the three residues critical for the activity of angiogenin—Lys-40, His-13, and His-114—and to a lesser extent its single tryptophan, Trp-89.

Human angiogenin, a protein that induces neovascularization (Fett et al., 1985), is ~35% identical in sequence with pancreatic ribonuclease (RNase)[1] (Strydom et al., 1985; Kurachi et al., 1985) and is ribonucleolytically active (Shapiro et al., 1986a; St. Clair et al., 1987; Rybak & Vallee, 1988). Placental ribonuclease inhibitor (PRI), a 50-kDa protein that is a tight-binding inhibitor of RNases (Blackburn et al., 1977; Blackburn & Moore, 1982), abolishes both the angiogenic and the ribonucleolytic activities of angiogenin (Shapiro & Vallee, 1987). The interaction between PRI and angiogenin is extremely tight (Lee et al., 1989a,b); the $K_i$ value is $7.1 \times 10^{-16}$ M, about 60 times lower than that for RNase A. The value of the apparent second-order rate constant of association of PRI with angiogenin is $1.8 \times 10^8$ $M^{-1}$ $s^{-1}$, close to the diffusion-controlled limit, while the value of the dissociation rate constant of the resultant complex is $1.3 \times 10^{-7}$ $s^{-1}$, yielding a half-life of about 60 days. This tight-binding interaction suggests that PRI may play a physiological role in the regulation of angiogenin (Shapiro & Vallee, 1987; Lee et al., 1989a,b).

The primary structure of PRI has been determined (Lee et al., 1988), and it is therefore possible to investigate the contact regions in angiogenin and PRI, with the eventual aim of designing and synthesizing angiogenin inhibitors with binding properties similar to those of PRI. In order to help define the angiogenin contact region, we have examined the binding of angiogenin derivatives to PRI. We find that modification or mutagenesis of three residues critical to the ribonucleolytic activity of angiogenin—Lys-40, His-13, and His-114—and the single tryptophan of angiogenin, Trp-89, each results in a weakening of the interaction with PRI, suggesting that these residues participate in the contact with PRI. The interaction of Lys-40 appears to be of critical importance: substitution of glutamine for this residue increases the $K_i$ value 1300-fold.

## Experimental Procedures

*Materials.* UpA was obtained from Sigma Chemical Co. Butanedione was from Aldrich Chemical Co. Human angiogenin used for chemical modification studies was purified from conditioned media of baby hamster kidney cells genetically engineered to synthesize it (Kurachi et al., 1988). Concentrations of angiogenin and its derivatives were determined by amino acid analysis. The sources of all other materials, including proteins, have been described (Shapiro et al., 1987; Lee et al., 1989a).

*Site-Specific Mutagenesis.* An angiogenin mutant in which Lys-40 was changed to Gln by site-specific mutagenesis was treated with *Aeromonas* aminopeptidase to remove the N-terminal Met as described (Shapiro et al., 1988a), thereby generating the natural pyroglutamyl N-terminus. Native angiogenin was prepared in an analogous manner.

*Chemical Modifications.* All reactions were performed in the dark at 25 °C. For C18 HPLC, a Synchropak RP-P reversed-phase HPLC column (250 × 4.1 mm; Synchrom, Inc.) and a Waters Associates liquid chromatography system equipped with a 254/214-nm detector and a Hewlett Packard 3390A integrator were employed. C18 HPLC solvents were as follows: A, 0.1% TFA; B, 2-propanol/acetonitrile/$H_2O$ (3:2:2 v/v) containing 0.08% TFA. The flow rate was 1 mL/min unless otherwise noted.

(*A*) *Histidine.* Angiogenin, 410 μg, was treated with 30 mM bromoacetate in 50 mM sodium acetate, pH 5.5, for 4 h

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[1] Abbreviations: RNase, pancreatic ribonuclease; RNase A, bovine pancreatic ribonuclease A; PRI, placental ribonuclease inhibitor; UpA, uridylyl(3′–5′)adenosine; FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, dinitrophenyl; DMS, dimethyl sulfide, SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; CM, carboxymethyl; C18, octadecylsilane; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid.

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(Shapiro et al., 1987) and applied to a Synchropak CM300 cation-exchange HPLC column (250 × 4.1 mm; Synchrom, Inc.). Derivatives were eluted with a 40-min linear gradient from 0.1 to 0.3 M NaCl in 20 mM sodium acetate, pH 4.0, at a flow rate of 1 mL/min. Fractions of the central peak were pooled and desalted by C18 HPLC prior to kinetic analysis.

(*B*) *Tryptophan.* Angiogenin, 620 μg, was oxidized by means of DMSO/HCl as described (Lee et al., 1989a) except that the times of the DMSO/HCl and DMS/HCl reactions were decreased to 20 and 30 min, respectively, and elution of the derivatives from C18 HPLC was achieved with a 27-min linear gradient from 30 to 42% solvent B at a flow rate of 0.8 mL/min.

(*C*) *Lysine.* Two different modifications of the lysines of angiogenin were performed. In one, 800 μg of angiogenin was dinitrophenylated with 3 mM FDNB in 50 mM Hepes, pH 8, for 1 h (Shapiro et al., 1989). The reaction mixture was applied to a C18 column, and derivatives were eluted with a 135-min linear gradient from 30% to 60% solvent B. In the other, 180 μg of angiogenin was reductively methylated with 2 mM formaldehyde and 10 mM sodium cyanoborohydride in 25 mM Hepes, pH 7.5, for 90 min (Shapiro et al., 1987). Derivatives were separated from reagent by C18 HPLC employing a 27-min linear gradient from 24 to 48% solvent B.

(*D*) *Arginine.* Angiogenin, 180 μg, was incubated with 20 mM butanedione in 25 mM Hepes, pH 8, for 90 min followed by separation from reagent by C18 HPLC using a 27-min linear gradient from 0 to 96% solvent B.

*Protein Structural Characterization.* Amino acid analysis was conducted using the Pico-Tag method (Bidlingmeyer et al., 1984). (Carboxymethyl)cysteine and tryptophan analyses were performed as described (Shapiro et al., 1986b, 1988b). Peptide mapping of tryptic digests of unmodified and modified angiogenin was performed as described (Shapiro et al., 1988a). Sequencing studies were carried out on a Beckman 890 sequencer upgraded to microsequencing status (Strydom et al., 1986).

*Inhibition Kinetics.* (*A*) *Association Rate Constant.* The apparent second-order association rate constants, $k_a$, for PRI and angiogenin derivatives were determined by examining the competition between the angiogenin derivative and RNase A for PRI as described (Lee et al., 1989a). PRI was added to a mixture of angiogenin derivative and RNase A, and the degree of partitioning of PRI between the two was determined by assay of free RNase A. Under the conditions of the experiment, angiogenin does not cleave UpA, the substrate employed, at a detectable rate (Shapiro et al., 1986a), and dissociation either of RNase A or of any the angiogenin derivatives from PRI is negligible. The following equation was employed (Lee et al., 1989a):[2]

$$\frac{k_{a,A}}{k_{a,R}} = \frac{\ln ([A]_T/[A]_F)}{\ln ([R]_T/[R]_F)}$$

where A and R refer to angiogenin derivative and RNase A, respectively, and T and F denote total and free concentration, respectively. The value of $k_{a,R}$ was taken to be $3.4 \times 10^8$ $M^{-1}$ $s^{-1}$ (Lee et al., 1989a). The substrate concentration, total RNase A concentration, and time between addition of PRI and substrate were 100 μM, 0.70 nM, and 60 s, respectively. The values of $k_{a,A}$ obtained were averages of at least six determinations.

(*B*) *Dissociation Rate Constant.* The dissociation rate constants, $k_d$, of the angiogenin derivative·inhibitor complexes were determined by first forming the complex, then adding a scavenger for free PRI, and subsequently assaying for free angiogenin derivative as a function of time, up to 10 days, as described (Lee et al., 1989b). The total derivative concentration was taken to be the free derivative concentration at $t = \infty$. The scavenger was RNase A, and the method of assay for free derivative was by cation-exchange HPLC as described (Lee et al., 1989b). The final concentration of angiogenin derivative was 0.70 μM. All samples were incubated in the dark.

For the angiogenin derivatives prepared by modification with formaldehyde/sodium cyanoborohydride or with butanedione, free derivative was assayed by injection onto a Mono S cation-exchange HPLC column (50 × 5 mm; Pharmacia) instead of a Synchropak CM300 column. Elution was achieved with either a 15-min linear gradient from 15 to 55% buffer B (formaldehyde/sodium cyanoborohydride) or a 10-min linear gradient from 15 to 85% buffer B (butanedione), where buffer A is 10 mM Tris, pH 7.8, and buffer B is 10 mM Tris, pH 8, containing 1 M NaCl. In all cases, free derivative was well resolved both from its complex with PRI and from free RNase A.

## Results

*$K_i$ Determinations.* Angiogenin binds to PRI with such an extremely low $K_i$, 0.71 fM, that it was necessary to determine both the association and dissociation rate constants in order to obtain the $K_i$ value (Lee et al., 1989b). Accordingly, the binding of angiogenin derivatives, modified at sites both within and outside the active site, has been analyzed in a similar manner using published methods (Lee et al., 1989a,b). Briefly, the apparent second-order rate constant of association is determined by competition between the angiogenin derivative and RNase A for PRI. From the known apparent association rate constant of RNase A and PRI, the corresponding value for the angiogenin derivative can be calculated. The dissociation rate constant is determined by first forming the angiogenin derivative·PRI complex. Release of free angiogenin derivative from this complex is then followed as a function of time in the presence of a 250-fold molar excess of RNase A as scavenger for free PRI.

*Lysine Modification.* C18 HPLC following treatment of angiogenin with formaldehyde and sodium cyanoborohydride results in a major peak with three minor shoulders eluting later (not shown). Amino acid analysis of the pooled material reveals the loss of 6.2 of 7 lysines. The apparent association rate constant of this modified angiogenin with PRI is only slightly decreased relative to that of unmodified angiogenin (Table I). The dissociation of this modified derivative from its complex with PRI does not follow first-order kinetics over the course of the experiment (56% dissociation, 3.3 days); the rate constant of dissociation decreases as a function of time (data not shown). However, the initial rate of dissociation is >100-fold higher than that for native angiogenin, and half of the complex has dissociated in only 2 days.

*Lys-40.* Lys-40 was changed to Gln by site-specific mutagenesis (Shapiro et al., 1989). The isolated mutant was treated with *Aeromonas* aminopeptidase to produce the natural pyroglutamyl N-terminus (Shapiro et al., 1988a) in >95% yield, as indicated by Edman degradation.

[2] We have shown previously (Lee et al., 1989a) that PRI binds to native angiogenin by a two-step mechanism:

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

where E is angiogenin and I is PRI. $k_a \simeq k_2/K_i$ when both [E] and [I] $\ll K_i$. In this experiment, the highest concentration of either protein is 2.8 nM, 190-fold lower than the $K_i$ value observed for native angiogenin, 530 nM, and likely much less than the $K_i$ value for all angiogenin derivatives.

Table I: Binding of Angiogenin Derivatives to PRI[a]

| reagent(s) | residue modified | $k_a \times 10^{-8}$ ($M^{-1}$ $s^{-1}$) | $k_d \times 10^7$ ($s^{-1}$) | $K_i$ (fM) | $K_{i,mod}/K_{i,unmod}$ |
|---|---|---|---|---|---|
| native (plasma)[b] | | 1.8 | 1.3 | 0.71 | |
| native (*E. coli*) | | 1.7 | 1.4 | 0.82 | 1.2 |
| mutagenesis[c] | Lys-40 | 0.61 | 570 | 930 | 1300 |
| bromoacetate | His-13;114[d] | 1.9 | 20 | 11 | 15 |
| DMSO/HCl | Trp-89 | 2.0 | 3.3 | 1.7 | 2.4 |
| FDNB | Lys-50 | 1.6 | 1.0 | 0.63 | 0.9 |
| FDNB | Lys-60 | 2.3 | 1.5 | 0.65 | 0.9 |
| $H_2CO/NaCNBH_3$ | Lys (6.2)[e] | 1.4 | *f* | | |
| butanedione | Arg (6.9)[e] | 0.71 | *f* | | |

[a] Conditions are 0.1 M Mes, pH 6, 0.1 M NaCl, and 1 mM EDTA, 25 °C. $k_a$ and $k_d$ are the association and dissociation rate constants, respectively. The subscripts mod and unmod refer to modified and unmodified angiogenin, respectively. [b] Lee et al. (1989a,b). [c] Lys-40 changed to Gln by site-specific mutagenesis (Shapiro et al., 1989). [d] Derivative carboxymethylated at a His-13:His-114 ratio of 1:1.8. [e] Number of residues modified indicated in parentheses. [f] Non-first-order dissociation kinetics are observed, but the initial rate of dissociation is >100-fold ($H_2CO$/$NaCNBH_3$) or >10-fold (butanedione) higher than that for native angiogenin.

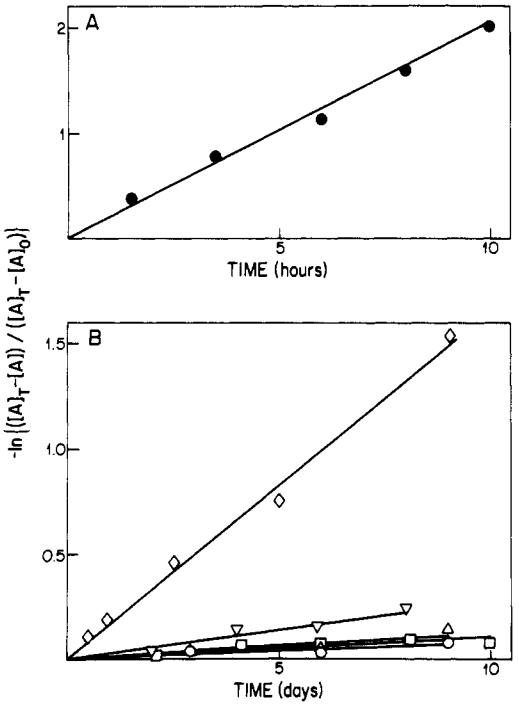


FIGURE 1: Determination of dissociation rate constants of (A) the Gln-40 angiogenin·PRI complex or (B) other angiogenin derivative·PRI complexes. The angiogenin derivative was incubated with 1.5 equiv of PRI for 20 min at 25 °C. Release of free angiogenin derivative, A, was then determined by HPLC after addition of 250 equiv of RNase A. The buffer was 0.1 M Mes, pH 6, containing 0.1 M NaCl, 1 mM EDTA, and 120 μM DTT. The dissociation rate constant is obtained from the slope of a plot of $-\ln\{([A]_T - [A])/([A]_T - [A]_0)\}$ vs $t$, where the subscripts T and 0 refer to the concentration of total angiogenin derivative and that observed several minutes after addition of scavenger, respectively. All concentrations are normalized to a control sample lacking PRI. The angiogenin derivatives are Gln-40 (●), CM-His-13;114 (◇), DMSO/HCl-oxidized Trp-89 (▽), DNP-Lys-50 (○), and DNP-Lys-60 (△). Data for native angiogenin expressed from *E. coli* are also shown (□).

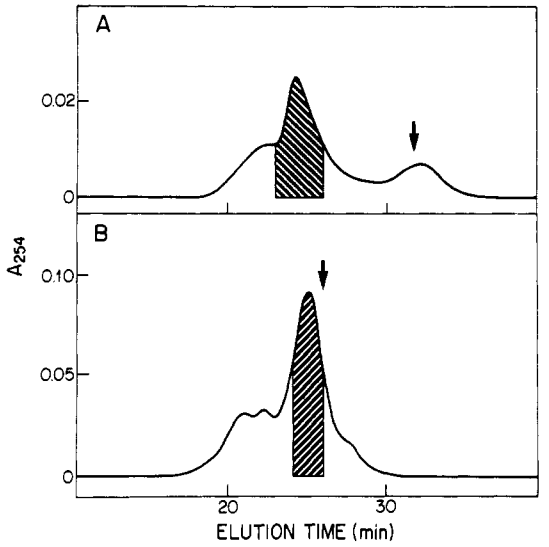


FIGURE 2: Purification of angiogenin derivatives modified by (A) bromoacetate or (B) DMSO/HCl. Hatched areas indicate the fractions taken for kinetic analysis. Arrows indicate elution positions of native angiogenin. (A) Angiogenin was incubated with 30 mM bromoacetate in 50 mM sodium acetate, pH 5.5, for 4 h at 25 °C followed by injection onto an Synchropak CM300 cation-exchange HPLC column. Elution was performed as described under Experimental Procedures. (B) Angiogenin was treated with 0.4 M DMSO in 8 M HCl for 20 min and then with 0.4 M DMS in 8 M HCl for 30 min at 25 °C. Each step was followed by filtration through Sephadex G-25. The final product was applied to a Synchropak RP-P C18 HPLC column. Elution was performed as described under Experimental Procedures.

The apparent association rate constant of this mutant with PRI is $6.1 \times 10^7$ $M^{-1}$ $s^{-1}$, 3-fold lower than that for native angiogenin. The dissociation rate constant of the mutant·PRI complex is $5.7 \times 10^{-5}$ $s^{-1}$ ($t_{1/2}$ = 3.4 h; Figure 1A), 440-fold higher than that for the native angiogenin·PRI complex ($t_{1/2}$ = 62 days). Therefore, the $K_i$ value for the binding of this mutant to PRI is 1300-fold higher than that for native angiogenin (Table I). Native angiogenin was prepared in an analogous manner from *Escherichia coli*, and its N-terminus was also >95% blocked. The parameters for PRI binding to this native angiogenin expressed from *E. coli* are essentially identical with those for native angiogenin obtained from human plasma (Table I).

*Lys-50 and Lys-60.* Angiogenin treated with FDNB can be resolved into several products, two of which are selectively derivatized at either Lys-50 or Lys-60, by C18 HPLC. The isolation and characterization of these derivatives are described elsewhere (Shapiro et al., 1989). Both derivatives bind to PRI with $K_i$ values within 10% of that of native angiogenin (Table I).

*His-13;114.* Attempts to separate monocarboxymethyl (mono-CM) from di-CM-angiogenin and unmodified angiogenin following treatment with bromoacetate were unsuccessful (R. Shapiro and S. Weremowicz, personal communication). These included cation-exchange HPLC (Mono S, Pharmacia) at pH 8 and 9.5 and reversed-phase HPLC (C18, Synchrom, Inc.). On the other hand, cation-exchange HPLC on a CM column at pH 4.0, near the p$K_a$ of the column's functional group, did separate the derivatives (Figure 2A). Amino acid analyses combined with expectations of the numbers of histidines modified from previous work (Shapiro et al., 1988b) indicate that the central peak (hatched in Figure 2A) is mono-CM-angiogenin while the earlier and later eluting

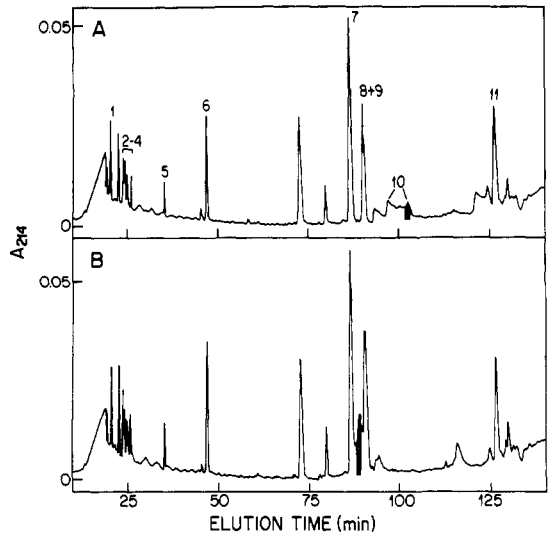


FIGURE 3: Fractionation of peptides from a tryptic digest of (A) native and (B) DMSO/HCl-modified angiogenin on a Beckman Ultrasphere IP (C18) HPLC column. Solvents A and B are defined under Experimental Procedures under Chemical Modifications. A 150-min linear gradient from 0 to 50% B at a flow rate of 0.8 mL/min was employed. Peptides 1 through 11 [see numbering system of Strydom et al. (1985)] for native angiogenin are indicated. Hatched areas indicate peptides compared for amino acid compositions.

peaks are di-CM-angiogenin and unmodified angiogenin, respectively. In addition, amino acid analysis of the mono-CM-angiogenin revealed the presence of 0.33 and 0.60 residues of $N^3$-CM-His and $N^1$-CM-His, respectively (see Discussion).

The apparent association rate constant of the mono-CM derivative with PRI is essentially unchanged compared to that of native angiogenin. The dissociation of its complex with PRI, despite the presence of a mixture of two mono-CM derivatives, is first order (Figure 1B). The dissociation rate constant is 15-fold higher ($t_{1/2}$ = 4.0 days) than that for native angiogenin; the resulting $K_i$ value is thus 15-fold higher (Table I).

*Trp-89.* The single tryptophan of angiogenin was oxidized with DMSO and HCl. The reported oxidation product of this procedure is oxindolealanine (Savige & Fontana, 1977). Chromatography of the derivatives on C18 HPLC revealed one major peak (hatched in Figure 2B). Protein in this peak was more than 99% uncleaved, as judged by SDS-PAGE under reducing conditions (Laemmli, 1970). Protein in all other peaks was cleaved substantially. Amino acid analysis of the major peak showed loss of the one tryptophan with no other changes in composition. The tryptophan fluorescence of this derivative was abolished (<5%).

Tryptic digestion of the oxidized angiogenin followed by fractionation indicated the presence of peptides indistinguishable in elution time from peptides 1–11 of native angiogenin [see numbering system of Strydom et al. (1985)] except for peptide 10, the tryptic peptide containing Trp-89 (Figure 3). A new peptide (hatched in Figure 3B) eluted earlier than peptide 10.[3] This peptide had an amino acid composition essentially the same as that of peptide 10, except for the absence of tryptophan (data not shown). These results are consistent with modification solely at Trp-89.

The association rate constant of this oxidized angiogenin with PRI is similar to that for unmodified angiogenin, but the dissociation rate constant of the complex is 2.5-fold higher (Figure 1B). The resulting $K_i$ value is 2.4-fold higher than that for unmodified angiogenin (Table I).

*Arginines.* Angiogenin treated with butanedione resulted in a single sharp peak on C18 HPLC employing a steep gradient to separate product from reagent (not shown). Amino acid analysis of the material indicated loss of 6.9 of 13 arginines. The apparent association rate constant of this modified angiogenin with PRI is 2.5-fold lower than that for unmodified angiogenin (Table I). The dissociation of its complex with PRI does not follow first-order kinetics over the course of the experiment (20% dissociation, 8.6 days); the dissociation rate constant decreases as a function of time (data not shown). In addition, the elution time of butanedione-modified angiogenin on the Mono S cation-exchange column employed for assay of dissociated derivative shifts toward that of native angiogenin during the course of the experiment, suggesting reversal of the modification. Nevertheless, the initial rate of dissociation is >10-fold higher than that for native angiogenin.

## DISCUSSION

Determination of the angiogenin·PRI contact region is necessary both to understand the basis of the extremely low $K_i$ value of the interaction and to design angiogenin inhibitors. We have therefore examined the effect of amino acid modification of angiogenin on its binding to PRI. The following were examined: (i) the active-site residues, Lys-40, His-13, and His-114, since PRI is a competitive inhibitor of RNase A and likely one of angiogenin (Lee et al., 1989b); (ii) the single tryptophan, Trp-89, because previous fluorescence data suggested that its environment undergoes a change in the PRI complex (Lee et al., 1989a); and (iii) lysines and arginines because they might be involved in binding a very basic protein, such as angiogenin (p$I$ > 9.5; Fett et al., 1985), to an acidic one, PRI (p$I$ = 4.7; Blackburn et al., 1977).

Substitution of a Gln for the active-site Lys-40 in angiogenin weakens the interaction 1300-fold (Table I); the effect corresponds to a free energy change of 4.3 kcal/mol. This is in the range of experimental values, ~2–6 kcal/mol, for the removal of either a salt-bridge partner or a hydrogen-bond partner to a charged group (Fersht, 1972, 1987; Fersht et al., 1985; Lowe et al., 1987; Wells et al., 1987), suggesting the existence of a similar situation in this instance. This, in turn, would imply the presence of an anionic partner to Lys-40 in PRI. The marked effect of NaCl concentration on PRI kinetics supports this proposition: an increase in the NaCl concentration from 0.1 to 1 M decreases the apparent association rate constant of PRI with angiogenin 100-fold (Lee et al., 1989a) and weakens the binding of PRI to RNase A 20 000-fold (Lee et al., 1989b). The magnitude of the effect of this single residue substitution on the binding of angiogenin to PRI is similar to that observed with certain contact residue substitutions in ovomucoids (e.g., Asp → Ala) on their binding to serine proteases (Empie & Laskowski, 1982).

The consequences of modification of either of two other active-site residues of angiogenin, His-13 and His-114, suggest that these are also involved in the interaction. Amino acid composition data (see Results) indicate that the reaction product examined is a mixture of angiogenins singly carboxymethylated either at His-13 or at His-114 in a ratio of 1:1.8, modified at the $N^3$- and $N^1$-positions, respectively (Shapiro et al., 1988b), which bind 15-fold more weakly to PRI than does native PRI (Table I). The change in $K_i$ value is due entirely to a change in the dissociation rate of the derivative·PRI complex, and this dissociation follows first-order kinetics for at least 80% of the process (Figure 1B). Therefore,

[3] This new peptide may be composed of more than one peak (see minor peaks/shoulders at 88, 90, and 94 min in Figure 3B). This is most likely due to the same reversible conformational change that characterizes peptide 10 (Figure 3A). Under the conditions employed, heterogeneity in the tryptophan oxidation product is unlikely (Savige & Fontana, 1980).

this suggests that the two derivative·PRI complexes dissociate at similar rates, which are significantly faster than that of the native angiogenin·PRI complex. This, in turn, suggests that both histidines contact PRI. Alternatively, the observed effects could reflect other aspects of the modification, e.g., the introduction of a negative charge or the prevention of access of PRI to Lys-40.

Lys-40, His-13, and His-114 are all critical for the ribonucleolytic activity of angiogenin (Shapiro et al., 1987; Shapiro et al., 1989). They are fully conserved in all pancreatic RNases (Beintema et al., 1986) and correspond to the catalytic triad of Lys-41, His-12, and His-119 of RNase A (Strydom et al., 1985). In the computed three-dimensional structure of angiogenin, these three residues are in close proximity to one another and reside in the proposed active-site cleft (Palmer et al., 1986). The view that these residues contact PRI, i.e., that PRI binds to angiogenin's active site, is consistent with previous kinetic studies which suggested that PRI is a competitive inhibitor of RNase A and, thus, likely one of angiogenin as well (Lee et al., 1989b).

A number of observations previously suggested that the single tryptophan of angiogenin, Trp-89, may also be part of the contact region. (i) Tryptophan fluorescence is enhanced 50% upon angiogenin·PRI complex formation and is abolished when Trp-89 is first oxidized by DMSO and HCl (Lee et al., 1989a). (ii) In the PRI complex Trp-89 becomes less accessible to acrylamide (Lee et al., 1989a). (iii) Tyr-92 in RNase A substitutes for Trp-89 (Strydom et al., 1985); the former is adjacent to Lys-91, which is fully protected from amidination in the RNase A·PRI complex (Blackburn & Gavilanes, 1982).

The present studies indicate that oxidation of Trp-89 by DMSO and HCl weakens the $K_i$ value for binding by PRI 2.4-fold by increasing the dissociation rate of the derivative·PRI complex (Table I). This increase is significantly less than that observed when any of the three active-site residues of angiogenin are modified. Still, the $K_i$ value is greater than that for the native angiogenin·PRI complex and, in light of the previous work, suggests that Trp-89 is a contact residue, although a less critical one than those at the active site. In the computed three-dimensional structure of angiogenin, Trp-89 is close to and on the same face as the active site of angiogenin (Palmer et al., 1986).

Modification of lysine and arginine residues by reductive methylation (6.2 residues) and by reaction with butanedione (6.9 residues), respectively, weakens the interaction between angiogenin and PRI (Table I) and suggests that these types of residues are involved in the binding to PRI. The dissociation of the products of either reaction from PRI does not follow first-order kinetics. This is likely due in large part to heterogeneity of the modified species, generating heterogeneity of dissociation rate constants. With regard to the lysine residues, the data presented indicate that Lys-40 is part of the contact region but two residues not thought to be in the active site, Lys-50 and Lys-60, are not. Angiogenin has 13 arginines, most of them in positions of nonconservative substitution in relation to RNase A (Strydom et al., 1985), and it remains to be seen which of these are involved in the interaction.

Given the homology in the primary structures of angiogenin and RNases (Strydom et al., 1985), the present results for the interaction of PRI with angiogenin may bear upon that of PRI with RNases in general. It has been suggested that the binding site of RNase A for PRI is extensive (Blackburn & Moore, 1982). In the three-dimensional structure of RNase A, the contact points can be grouped as follows: (A) Lys-7, Lys-41, Pro-42, Val-43, Lys-91, Tyr-92, Pro-93; (B) Lys-31, Lys-37; (C) Lys-61 and adjacent residues. Many of these residues are identical or similar in angiogenin (Shapiro & Vallee, 1987). Of relevance here are the angiogenin counterparts of RNase A Lys-41 (Lys-40), Tyr-92 (Trp-89), and Lys-61 (Lys-60).

Comparison of the work on RNase A with the present results on angiogenin reveals several apparent differences. (1) Carboxymethylation of Lys-41 of RNase A weakens the interaction with PRI only 10-fold (Blackburn & Gavilanes, 1980), whereas substitution of Gln for Lys-40 of angiogenin, a seemingly less drastic modification in terms of charge and steric effects, weakens it 1300-fold. (2) Carboxymethylation of His-12 and His-119 of RNase A strengthens the interaction 3-fold (Blackburn & Jailkhani, 1979; Blackburn & Gavilanes, 1980), whereas carboxymethylation of His-13 and His-114 of angiogenin weakens it 15-fold. (3) Lys-61 of RNase A is fully protected from amidination by PRI (Blackburn & Gavilanes, 1982), whereas dinitrophenylation of Lys-60 of angiogenin does not affect its interaction with PRI.

With respect to the three residues discussed in (1) and (2), we note that previous conclusions were based on experiments that determined relative $K_i$ values by examining the competition between RNase A derivatives modified at these residues and unmodified RNase A for PRI at equilibrium (Blackburn & Jailkhani, 1979; Blackburn & Gavilanes, 1980). However, in these experiments, the derivative was bound to PRI before addition of RNase A, and the time required to reach equilibrium (up to several hours; Lee et al., 1989b) was likely underestimated. As a consequence, the effect of modification on the strength of binding to PRI was probably underestimated as well. The present approach involves determination of both the association and dissociation rate constants for the angiogenin derivative·PRI interaction. Thus, attainment of equilibrium is not necessary for determinations of $K_i$ values. It remains to be seen whether the differences observed between angiogenin and RNase A are solely due to the different methodologies employed or to some inherent difference in their modes of interaction with PRI.

With respect to Lys-60 (3), we note that this residue is in a region of the primary structure of the molecule which contains two deletions and other major changes in relation to the primary structure of RNase A (Strydom et al., 1985). Therefore, in this instance, the two lysines (60 of angiogenin, 61 of RNase A) may differ in their placement in the three-dimensional structure of the two proteins, and this may account, in part, for the apparent discrepancy. Of interest here is that an angiogenin/RNase A hybrid molecule, in which the RNase A sequence Ser-59 to Tyr-73 substitutes for Glu-58 to Arg-70 in angiogenin, binds as tightly to PRI as does native angiogenin (Harper & Vallee, 1989).

Our studies indicate that PRI contacts a discrete region of the angiogenin molecule, which may be smaller than that suggested by Blackburn and co-workers for RNase A (Blackburn & Moore, 1982), which minimally encompasses Lys-40, His-13, His-114, and Trp-89. The involvement of the first three residues, all part of the active site of angiogenin, indicates that PRI binds to that site. The further elucidation of the contact region of angiogenin and of its complement, i.e., that of PRI, should provide the basis for the design of novel angiogenin inhibitors based on PRI and be facilitated by our recent cloning and sequencing of the PRI cDNA (Lee et al., 1988).

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**Registry No.** Lys, 56-87-1; Gln, 56-85-9; His, 71-00-1; PRI, 39369-21-6.

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