Identification of Functional Arginines in Human Angiogenin by Site-Directed Mutagenesis†

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ABSTRACT: Chemical modifications of human angiogenin had suggested that arginines are essential for its ribonucleolytic activity [Shapiro, R., Weremowicz, S., Riordan, J. F., & Vallee, B. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8783–8787]. Each of the six arginines within or near angiogenin's catalytic or cell-binding sites—i.e., those at positions 5, 31, 32, 33, 66, and 70—was therefore mutated to alanine. Two of these residues, Arg-5 and Arg-33, indeed play a role, albeit noncrucial, in enzymatic activity, although neither one is implicated in the abolition of activity by arginine reagents. R5A-angiogenin, while nearly fully active toward dinucleotides, is one-fourth as active as angiogenin toward tRNA, suggesting that Arg-5 may participate in the binding of peripheral components of the substrate. In contrast, the activity of R33A-angiogenin toward both polynucleotide and dinucleotide substrates is reduced similarly, reflecting a decrease in k_{cat}. These results, together with its position in the calculated three-dimensional structure of angiogenin, imply an indirect role for Arg-33 in catalysis. Three arginines are important for angiogenesis: mutation of Arg-5, Arg-33, or Arg-66 dramatically reduces the angiogenic potency of angiogenin on the chicken embryo chorioallantoic membrane. Arg-66 lies within a segment previously proposed to be part of a cell-surface receptor binding site. Arg-5 and Arg-33 are outside of this site as defined at present, and the decreased angiogenicity of R5A- and R33A-angiogenin may be a consequence of their reduced ribonucleolytic activities. Arginines are also important for the interaction of angiogenin with human placental angiogenin/ribonuclease inhibitor, since mutation of Arg-5 and Arg-32 increases K_i by 50-fold and 4-fold, respectively, largely reflecting an increased rate of dissociation of the complex.

Human angiogenin induces neovascularization in the chicken embryo chorioallantoic membrane (CAM)[1] and the rabbit cornea and meniscus (Fett et al., 1985; King & Vallee, 1991). Its sequence homology to the pancreatic RNase superfamily (Strydom et al., 1985; Kurachi et al., 1985) led to the recognition of its unusual ribonucleolytic activity (Shapiro et al., 1986b) and suggested an approach to the study of its structure–function relationships based on the methods employed previously with bovine RNase A [see Richards and Wyckoff (1971) and Blackburn and Moore (1982)]. Thus, the chemical modifications which had demonstrated His-12, His-119, and Lys-41 of RNase to be critical for enzymatic activity were applied to angiogenin (Shapiro et al., 1986b, 1987). Bromoacetate at pH 5.5 was found to react specifically with His-13 and His-114 (Shapiro et al., 1988b), residues analogous to the two active-site histidines in RNase A. Indeed, the simultaneous decrease in both ribonucleolytic and angiogenic activities accompanying this modification was the first evidence that both of these depend minimally on the integrity of the catalytic apparatus known for RNases. This has been confirmed by several subsequent investigations (Shapiro & Vallee, 1987, 1989; Shapiro et al., 1989). Chemical modification of lysines also abolishes the enzymatic activity of angiogenin (Shapiro et al., 1987), and in this case, the crucial residue was identified by site-directed mutagenesis as Lys-40, corresponding to Lys-41 of RNase A (Shapiro et al., 1989).

Chemical modification also implicated Arg-39 and Arg-85 in the catalytic activity of RNase A (Takahashi, 1968). However, in contrast with the active-site histidines and lysine, neither one of these is present in angiogenin. Quite the same, chemical modifications with arginine reagents pointed to 1 or more of the 13 arginines in human angiogenin as essential for ribonucleolytic activity (Shapiro et al., 1987), although lack of reagent specificity precluded identification of any particular critical residue(s). Therefore, site-directed mutagenesis has again been employed for this purpose.

The selection of residues for mutation was based on the three-dimensional structure of angiogenin calculated from the X-ray coordinates of RNase A (Palmer et al., 1986). Only six arginines—those at positions 5, 31, 32, 33, 66, and 70—are located within or near the region corresponding to the active center of RNase (Figure 1). All of these are also of interest in relation to other aspects of angiogenin's function: Arg-66 and -70 lie within the proposed cell-surface receptor binding site (Hallahan et al., 1991), and Arg-5, -31, -32, and -33 are in or near a region that interacts with human placental RNase inhibitor (PRI), a tight-binding inhibitor of angiogenin (Shapiro & Vallee, 1987; Lee et al., 1989b; Lee & Vallee, 1989a). The present report examines the effects of mutating each of these six arginines to alanine in terms of ribonucleolytic and angiogenic activities, and PRI binding.

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[1] Abbreviations: CAM, chorioallantoic membrane; PRI, human placental RNase inhibitor; R5A, R31A, R32A, R33A, R66A, and R70A, angiogenin mutants in which Arg-5, Arg-31, Arg-32, Arg-33, Arg-66, or Arg-70, respectively, has been changed to alanine; PCR, polymerase chain reaction; C18, octadecylsilane; HPLC, high-performance liquid chromatography; CpA, cytidylyl-3′,5′-adenosine; UpA, uridylyl-3′,5′-adenosine; CpG, cytidylyl-3′,5′-adenosine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ARH-III, Met-(−1) angiogenin in which residues 8–22 have been replaced by amino acids 7–21 of RNase A.

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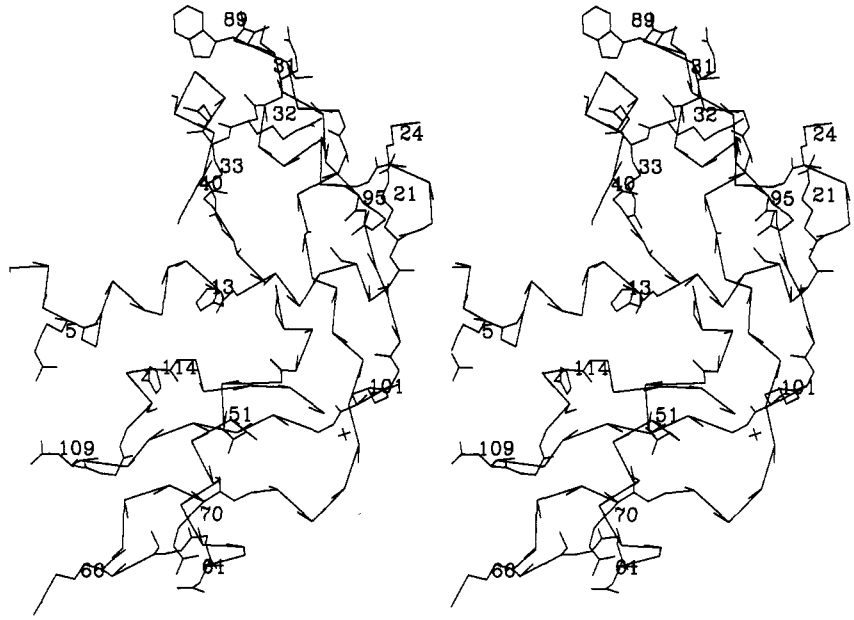

FIGURE 1: Stereoview of the structure of angiogenin calculated from the X-ray coordinates of RNase A by energy-minimization procedures (Palmer et al., 1986). The side chains of Arg-5, -21, -24, -31, -32, -33, -51, -66, -70, -95, and -101, His-13, Lys-40, and His-114 in the enzymatic active site, Asn-61 and Asn-109 in the putative cell-binding site, and Trp-89 are shown.

EXPERIMENTAL PROCEDURES

Met-(-1)[2] human angiogenin was obtained from a recombinant expression system in *Escherichia coli* (Shapiro et al., 1988a). Dr. M. D. Bond kindly provided the human angiogenin/bovine RNase A hybrid protein ARH-III (Bond & Vallee, 1990a) and bovine angiogenin. The latter was isolated from serum as described (Bond & Vallee, 1988) except that an additional chromatography on UTP–agarose was introduced in order to remove all traces of extraneous RNases. Protein concentrations were determined by amino acid analysis (PicoTag: Waters Associates). Oligonucleotides were synthesized on a Biotix Model 102 DNA synthesizer. Sources of other materials are listed elsewhere (Shapiro et al., 1986a,b, 1987, 1988a).

Oligonucleotide-Directed Mutagenesis. Genes encoding Met-(-1) R31A and Met-(-1) R33A mutant angiogenins were prepared by the method of Kunkel (1985) as described (Shapiro et al., 1988a). The mutagenic oligonucleotides were pTCGATTATGGCACGCCGTGGG (R31A) and pATGAGACGCGCTGGGTTAACT (R33A). Mutant genes were excised from double-stranded M13mp18 with *Kpn*I and *Eco*RI and inserted into *Kpn*I/*Eco*RI-digested expression vector pAng2 (Shapiro et al., 1988a) by standard procedures. Genes encoding R5A, R32A, R66A, and R70A were obtained by the PCR-based method of Ho et al. (1989) as described (Hallahan et al., 1992). The complementary mutagenic primers were pAGGACAACTCGGCCTATACACATT and pAATGTGTATAGGCCGAGTTGTCCT (R5A), pCGATTATGAGAGCCCGTGGGTTAA and pTTAACCCACGGGCTCTCATAATCG (R32A), pGTAACCCGCATGCCGAAAACCTGC and pGCAGGTTTTCGGCATGCGGGTTAC (R66A), pCGAAAACCTGGCCATCAGCAAGTC and pGACTTGCTGATGGCCAGGTTTTCG (R70A). The 5′-flanking primer in all instances was pAACTAGTACGCAAGTTCA, corresponding to a sequence in pAng2 upstream from the *Kpn*I site of the *trp* promoter. The 3′-flanking primer was pTCGACGGATCCCCGGGAATTC, corresponding to a sequence in the 3′-noncoding region of pAng2 that contains the *Eco*RI site. The template for the first round of PCR extension was the plasmid pAng3, which is identical to pAng2 except that the initiator methionine codon has been replaced by a DNA sequence encoding the *E. coli phoA* signal peptide (GTGAAACAAAGCACTATTGCACTGGCACTATTACCGTTACTGTTTACCCCTGTGACAAAAGCC) (Dodt et al., 1986). The final PCR product was digested with *Kpn*I and *Eco*RI and inserted into *Kpn*I/*Eco*RI-digested plasmid pAng1 (Shapiro et al., 1988a) by standard procedures. The DNA for each of the six mutants was sequenced in its entirety in order to rule out any spurious mutations.

Expression and Isolation of Mutant Proteins. Met-(-1) R31A and Met-(-1) R33A were expressed and purified to homogeneity as described (Shapiro et al., 1988a). The remaining mutant proteins were obtained from a modified expression system utilizing the vector pAng3, which produces the <Glu-1 protein. Three-milliliter cultures of *E. coli* W3110 harboring the expression plasmid were grown in LB broth containing 50 μg/mL ampicillin at 37 °C until $A_{600} > 2.0$ (usually ~8 h). Each culture was then added to 1 L of M9 medium (Sambrook et al., 1989) supplemented with 0.4% casamino acids, 0.4% glucose, 50 μg/mL ampicillin, and 20 μg/mL indole-3-acrylic acid and grown overnight with shaking at 37 °C. Fresh glucose, casamino acids, and indoleacrylic acid were then added to final concentrations of 0.2%, 0.2%, and 20 μg/mL, respectively, and the cultures were incubated at 37 °C for an additional 2 h. Cells were collected, resuspended, and treated with lysozyme as described (Shapiro et al., 1988a) except that the resuspension buffer was 20 mM Tris, pH 9.0, containing 10% sucrose, 2.5 mM phenylmethanesulfonyl fluoride, 10 mM *o*-phenanthroline (Sigma), and 0.25 mM benzamidine (Sigma). The mixuture was sonicated at 4 °C for 2 cycles of 15 s each with a Branson Model 350 sonifier, power setting 7 on "pulse" (70% on per second), and centrifuged at 35000*g* for 20 min at 4 °C. The supernatant was loaded onto a column of S-Sepharose (50

[2] Met-(-1) angiogenin is equivalent to the natural <Glu-1 protein with respect to angiogenic activity on the CAM and ribonucleolytic activity toward tRNA, dinucleotides, and 28S and 18S rRNA (Shapiro et al., 1988a).

mL/L of culture) which had been equilibrated with 50 mM Tris/100 mM NaCl, pH 8.0, at 4 °C, and the resin was washed with the same buffer until $A_{280} < 0.05$. Bound material was eluted with 50 mM Tris/1 M NaCl, pH 8.0, then concentrated by ultrafiltration, and chromatographed on a Mono S cation-exchange column as described (Shapiro et al., 1986b, 1988a). Fractions containing angiogenin derivatives were diluted with an equal volume of 0.4 M potassium phosphate, pH 7.2, incubated for 20 h at 37 °C to convert the N-terminal glutamine to pyroglutamic acid, and then chromatographed on a Synchropak C18 HPLC column (Shapiro et al., 1988a). Peak fractions were diluted 1:1 with water, lyophilized, and reconstituted in water. Precautions were taken to ensure that preparations were not contaminated by unmodified angiogenin (Shapiro & Vallee, 1989).

Structural Characterization. Amino acid analyses and N-terminal sequencing were performed as described (Strydom et al., 1985). Tryptic digests of native proteins (Shapiro et al., 1988a) were chromatographed on a Synchropak RP-P C18 HPLC column, and the unretained fraction was rechromatographed on a Beckman Ultrasphere C18 column (Hallahan et al., 1992).

Angiogenesis Assays. Angiogenic activity was assessed on the chick embryo CAM (Knighton et al., 1977; Fett et al., 1985). The numbers of positive and negative responses for each sample from multiple sets of assays were combined, and χ^2 were calculated from an outcome contingency table by comparing the test sample with a water control; the associated probabilities, p, were then obtained. A value of $p < 0.05$ identifies a sample as active (Fett et al., 1985).

Chemical Modification and Assay of Ribonucleolytic Activity. Reactions of angiogenin or angiogenin mutants (24 μM) with 10 mM *p*-hydroxyphenylglyoxal were performed in the dark at 27 °C in 0.1 M Hepes, pH 8.0. At various times, 5- or 10-μL aliquots were added to 235 or 230 μL, respectively, of 40 mM Hepes/40 mM NaCl, pH 7.0, containing 100 μg of RNase-free bovine serum albumin (Worthington) at 4 °C. After the last aliquot had been taken, 60 μL of 10 mg/mL tRNA was added to each tube, and the mixtures were incubated at 37 °C for 4 h. Perchloric acid precipitations and absorbance measurements at 260 nm were performed as described (Shapiro et al., 1987). Activity was quantitated by comparison with standard curves obtained using 0.1, 0.2, 0.4, 0.6, and 1.0 μg of unmodified angiogenin, R31A, R32A, R66A, and R70A; 0.3, 0.6, 1.2, 1.8, and 3.0 μg of R5A; or 0.7, 1.4, 2.8, 4.2, and 7.0 μg of R33A. Absorbance readings were corrected for any contribution of the modification reagent. The activity of modified angiogenin (2.4 μM) toward CpA was measured by HPLC quantitation of substrate and products after 4 h at 37 °C in 33 mM Mes/33 mM NaCl, pH 5.9, as described (Shapiro et al., 1986a,b).

Interaction of Angiogenin Derivatives with Human PRI. Apparent second-order rate constants for association were determined by a competition assay (Lee et al., 1989a) as described previously (Shapiro et al., 1989). Briefly, RNase A (6.8 nM) was mixed with 0.6–2.2 equiv of angiogenin derivative, PRI (6.8 nM) was added, and after 15 s, the concentration of free RNase A was quantitated by adding the dinucleotide CpG and measuring its rate of cleavage spectrophotometrically. Rate constants for dissociation of the complexes of PRI with mutant angiogenins were measured by HPLC quantitation of the free derivative at various times after addition of RNase A to act as a scavenger for free PRI (Lee et al., 1989b; Shapiro et al., 1989). Aliquots were chromatographed after 1 min and 22, 31, 46, 71, and 94 h for R5A and after 1–5 min and 1, 2, 3, 4, and 6 days for the remaining samples. Dissociation of all complexes was biphasic, with a relatively rapid release of up to 25% of the mutant protein followed by a slower dissociation of the remainder. Similar biphasic dissociation has been noted previously for the complexes of PRI with other angiogenin mutants (Shapiro et al., 1989; Shapiro & Vallee, 1989), and was observed for the PRI·angiogenin complex in a parallel experiment. [See Shapiro and Vallee (1991) for a discussion of possible explanations of this phenomenon.] Rate constants were calculated by treating the slower phase as a first-order process. The concentrations of angiogenin derivative in control samples lacking PRI did not change significantly over the course of the experiment. Incubation mixtures lacking scavenger contained no free mutant protein after 6 days.

RESULTS

Expression of the phoA Signal Peptide–Angiogenin Fusion Protein. The bacterial expression system employed previously for the preparation of angiogenin and its mutant derivatives (Shapiro et al., 1988a) produces the Met-(–1) protein as cytoplasmic inclusion bodies, which are solubilized under reducing conditions and reoxidized to the folded product. This procedure routinely generates 1–2 mg of angiogenin/L of culture, but the yields of mutant derivatives are highly variable: in earlier studies, they ranged from 5 mg/L for K40Q-angiogenin (Shapiro et al., 1989) to as little as 20 μg/L for D116H-angiogenin (Harper & Vallee, 1988). Indeed, repeated attempts to produce R5A for the present work by this method were unsuccessful: expression was undetectable by immunoblotting, and <2 μg of product could be isolated per liter. A modified expression system was therefore devised in which the *E. coli pho*A signal sequence was appended to the protein N-terminus. This should allow the product to be secreted into the periplasm in soluble form, minimizing contact with cytoplasmic proteases and bypassing the lengthy and potentially inefficient refolding steps required previously.

This system was first tested for expression of the natural protein in *E. coli* harboring the altered expression plasmid pAng 3: several milligrams per liter of angiogenin was produced as assessed by immunoblots. Despite the periplasmic targeting sequence, less than 5% of the protein was released from the cells after disruption of the outer membrane by standard procedures (Sambrook et al., 1989). Following complete cell lysis, however, virtually all of it was present in the soluble fraction which therefore served as the starting material for the isolation of angiogenin. S-Sepharose, Mono S, and C18 chromatography and phosphate treatment gave a final product (1.5 mg/L) that comigrated with the native protein during SDS–PAGE. The product was refractory to Edman degradation, indicating that the N-terminal glutamine had cyclized to the pyroglutamic acid characteristic of authentic angiogenin. The ribonucleolytic and angiogenic activities of the final preparation were indistinguishable from those of native angiogenin, as was its amino acid composition.

Preparation of Angiogenin Mutants. Genes encoding Arg→Ala mutants were prepared by two different methods of oligonucleotide-directed mutagenesis (see Experimental Procedures). Four mutant derivatives, R5A, R32A, R66A, and R70A, were expressed as chimeric proteins carrying the *pho*A signal sequence and purified from the soluble fraction as the <Glu-1 form, in each case with a yield of ~1 mg/L. Two others, R31A and R33A, were expressed as Met-(–1) derivatives and purified from inclusion bodies with yields of ~1 and 4 mg/L, respectively. All six mutants elute 1–4 min

Table I: Amino Acid Composition of Angiogenin Mutants Produced in *E. coli*[a]

amino acid	angiogenin[b]	mutants					
		R5A[c]	R31A[b]	R32A[c]	R33A[b]	R66A[c]	R70A[c]
Asx	14.0 (15)[d]	14.4	14.4	14.5	14.7	14.0	14.7
Glx	10.1 (10)	10.4	10.7	10.4	10.3	10.4	10.4
Ser	8.5 (9)	8.5	9.1	8.6	8.6	8.5	8.7
Gly	8.2 (8)	8.6	8.7	8.5	8.3	8.7	8.3
His	5.8 (6)	5.6	5.6	5.7	5.8	5.4	5.9
Arg	13.3 (13)	12.0	11.8	12.3	12.0	12.0	12.2
Thr	7.0 (7)	7.0	6.8	6.9	6.9	6.8	7.0
Ala	5.3 (5)	6.3	6.2	6.3	6.1	6.5	6.1
Pro	8.1 (8)	8.2	8.0	8.2	8.3	8.0	8.3
Tyr	3.9 (4)	3.8	3.8	3.9	3.9	3.6	3.9
Val	3.9 (5)	4.0	3.8	3.9	3.6	4.2	3.8
Met	2.1 (2)	1.3	2.2	1.2	2.2	1.2	1.2
Ile	6.6 (7)	6.2	6.3	6.4	6.3	6.3	6.5
Leu	6.2 (6)	6.2	6.0	6.2	6.1	6.2	6.1
Phe	5.0 (5)	4.8	4.8	4.9	4.9	4.9	5.0
Lys	6.9 (7)	7.7	6.9	7.3	7.0	8.2	7.0

[a] Duplicate analyses were performed as described (Strydom et al., 1985). Tryptophan and cysteine contents were not determined. [b] Met-(−1) mutant protein obtained from the expression system described by Shapiro et al. (1988a). [c] <Glu-1 mutant protein derived from the expression system for angiogenin containing signal peptide. [d] Values in parentheses from sequence (Strydom et al., 1985), plus Met-(1) residue.

Table II: Amino Acid Compositions of Selected Tryptic Peptides from Angiogenin Mutants[a]

amino acid	R5A	R31A[b]	R33A	R66A	R70A
Asx	2.64 (3)	1.80 (2)		2.50 (3)	0.93 (1)
Glx	3.05 (3)	2.17 (2)	1.13 (1)	1.02 (1)	1.09 (1)
Ser	1.03 (1)	2.92 (3)	1.98 (2)		0.96 (1)
Gly	1.21 (1)	0.13	3.02 (3)	1.00 (1)	
His	1.95 (2)		1.01 (1)	1.00 (1)	
Arg	1.01 (1)	1.99 (2)	1.01 (1)	1.08 (1)	
Thr	1.92 (2)	1.94 (2)	1.02 (1)		
Ala	2.00 (2)	1.10 (1)	0.95 (1)	0.93 (1)	1.09 (1)
Pro	1.13 (1)		3.95 (4)	0.93 (1)	
Tyr	1.84 (2)	0.96 (1)	1.02 (1)		
Val		1.08 (1)	0.11		
Met		1.14 (1)			
Ile		1.16 (1)			1.06 (1)
Leu	1.15 (1)		1.99 (2)	1.04 (1)	1.11 (1)
Phe	1.05 (1)	1.09 (1)		0.21	
Lys	0.98 (1)	0.98 (1)	0.96 (1)	0.12	0.76 (1)
pmol analyzed	110	90	96	145	89

[a] Cysteine and tryptophan contents were not determined. Each peptide shown corresponds to a new peak in the tryptic map of the mutant protein indicated. Numbers in parentheses represent compositions expected for R5A amino acids 1–21, R31A disulfide-linked amino acids 22–32 + 74–82, R33A disulfide-linked amino acids 33–40 + 83–95, R66A amino acids 61–70, and R70A amino acids 67–73. In native angiogenin, these sequences are found in peptides (T1 + T7), T9, T10, (T4a + T5), and (T3b + T5), respectively. [b] The amino acid composition of the R31A peptide has been corrected for the presence of 25 pmol of peptide T10.

earlier than angiogenin during Mono S HPLC, whereas their retention times during C18 HPLC are identical to that of the native protein. Final preparations were >98% pure as judged by SDS–PAGE.

Structural Characterization. The amino acid composition of each of the six mutant proteins demonstrates the alanine replacement but no other changes (Table I). Tryptic peptide maps differ from that of angiogenin only with respect to those peptides containing the mutated residues [see Strydom et al. (1985) for a description of angiogenin tryptic peptides]. Peptides T1 (residues 1–5) and T7 (residues 6–21) are absent from the map for R5A, and a new peptide eluting between T10 and T11 has the amino acid composition expected for residues 1–21 (Table II). Peptide T9 (amino acids 22–31 linked by a disulfide bond to amino acids 74–82) is absent from the tryptic map for R31A, and the height of the first peak of the peptide T10 doublet is increased. The amino acid composition of this material (Table II) reveals the presence

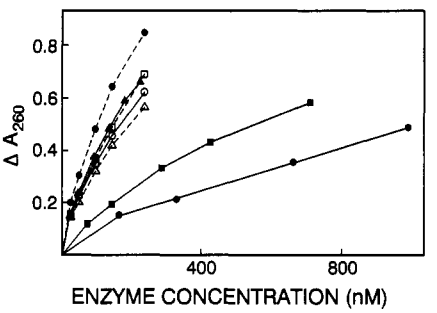

FIGURE 2: Cleavage of tRNA by angiogenin (▲—▲), R5A (■—■), R31A (□- - -□), R32A (●- - -●), R33A (●—●), R66A (△- - -△), and R70A (○—○). Assays were performed as described (Shapiro et al., 1987).

of the extended peptide T9 (residues 22–32 plus 74–82), together with 0.3 molar equiv of unmodified peptide T10. The peptide map of R32A is indistinguishable from that of angiogenin. No peptide containing residue 32 was recovered from either protein.

The peptide T10 doublet of R33A elutes 0.4 min later than the corresponding angiogenin peptide (amino acids 34–40 linked by a disulfide bond to amino acids 83–95). Its amino acid composition indicates the presence of residues 33–40 plus 83–95 consistent with the Arg→Ala replacement (Table II). Peptides T4a (amino acids 61–66) and T5 (amino acids 67–70) are absent from the tryptic map of R66A, and a new peak eluting between peptides T6 and T7 has the amino acid composition expected for residues 61–70 in the modified protein (Table II). The R70A digest lacks peptides T3b (amino acids 71–73) and T5 (amino acids 67–70). A new peptide eluting between T5 and T6 has the amino acid composition expected for residues 67–73 (Table II).

Enzymatic Activity. R33A is one-seventh as active toward tRNA as angiogenin, measured at substrate concentrations of both 2 (Figure 2) and 0.4 mg/mL. The former concentration is equal to or above and the latter is well below the apparent K_m value (Lee & Vallee, 1989b). The activity of R5A toward tRNA is also decreased, albeit less substantially (4-fold). The activities of the remaining four derivatives are more comparable to that of angiogenin [92% (R31A), 130% (R32A), 76% (R66A), and 83% (R70A)].

The activity of the mutant proteins toward the dinucleotides CpA, UpA, and CpG was also examined (Table III). The

Table III: Activities of Angiogenin and Mutant Derivatives toward Dinucleotides[a]

	k_{cat}/K_m, M^{-1} s^{-1}						
dinucleotide	angiogenin	R5A	R31A	R32A	R33A	R66A	R70A
CpA	14.3	8.6	17.7	17.6	2.5	12.5	8.1
CpG	4.0	3.8	5.0	4.7	0.69	4.3	3.3
UpA	0.92	0.55	2.0	0.86	0.22	0.64	0.39

[a] Incubations were performed in 33 mM Mes, pH 5.9, containing 33 mM NaCl and 0.1 mM substrate, 37 °C. Values for k_{cat}/K_m were determined by using an HPLC assay as described (Shapiro et al., 1986a,b).

Table IV: Effects of *p*-Hydroxyphenylglyoxal on the Ribonucleolytic Activity of Angiogenin and Angiogenin Mutants[a]

	activity, $v/v_c \times 100$						
time, min	angiogenin	R5A	R31A	R32A	R33A	R66A	R70A
15	30	30	36	56	42	38	42
30	11	12	19	29	14	18	13
60	1.5	2	12	7.5	2.9	3.5	1.9
90	0.5	0.7	9.4	3.6	<0.5	1.7	0.7

[a] Incubations were performed at 27 °C in 0.1 M Hepes, pH 8.0, with 10 mM *p*-hydroxyphenylglyoxal. Activities are listed as $v/v_c \times 100$, where v and v_c are reaction velocities in the presence and absence of modification reagent, respectively, and were determined with tRNA as substrate as described (Shapiro et al., 1987).

activity of R33A toward each of these substrates again is markedly lower than that of angiogenin; the extent of the decrease in all instances is similar to that observed with tRNA. Activities of the other five mutant proteins including R5A do not differ appreciably from that of native angiogenin.

Chemical Modification. Treatment of angiogenin with 10 mM *p*-hydroxyphenylglyoxal at pH 8.0 for 90 min at 27 °C reduces the activity toward tRNA by >98% (Shapiro et al., 1987). In order to ascertain whether or not any of the six arginines mutated are involved in this loss of activity, the rate of inactivation of each derivative by this reagent was measured (Table IV). R5A, R33A, R66A, and R70A are all inactivated at virtually the same rate as is angiogenin. The initial rate for R31A is similar, but decreases markedly after 30 min. Inactivation of R32A is slower throughout.

The effects of *p*-hydroxyphenylglyoxal on bovine angiogenin and the human angiogenin/bovine RNase A hybrid protein ARH-III were also determined (data not shown). Bovine angiogenin, like the human protein, contains 13 arginines, but only 6 are at corresponding positions [5, 21, 33, 66, 70, and 101 (Bond & Strydom, 1989)]. It is inactivated ~ 2.5-fold more slowly than its human counterpart. In ARH-III (Bond & Vallee, 1990a), angiogenin residues 8–22 are replaced with the RNase segment 7–21, thereby substituting Arg-21 by Ala. The rate of inactivation of this derivative is identical to that of human angiogenin.

In previous studies, only polynucleotide substrates have been used to monitor the effect of *p*-hydroxyphenylglyoxal treatment (Shapiro et al., 1987); a comparable rate and degree of inactivation was observed with each of them. To determine whether cleavage of small substrates is affected similarly, aliquots of a reaction mixture were assayed simultaneously with both tRNA and the dinucleotide CpA. Remarkably, when the level of activity measured with the polynucleotide substrate is virtually undetectable (<1%), the modified protein exhibits 58% of native activity toward CpA.

Angiogenic Activity of Mutant Angiogenins. Mutant angiogenins were tested for angiogenic activity at a dose of 10 ng/egg (Table V). R5A, R33A, and R66A are substantially less active than angiogenin, with a response statistically indistinguishable from that for control samples lacking angiogenin. However, R32A and R70A retain activities similar to that of angiogenin. Finally, R31A gives a highly significant positive response ($p = 0.0006$ compared to negative controls), but it is less active than the unmodified protein.

Table V: Angiogenic Activity of Angiogenin and Angiogenin Mutants[a]

sample	dose, ng	% positives, n[b]	p[c]
angiogenin	10	57 (35)	<0.0001
	1	48 (432)	<0.0001
R5A-angiogenin	10	23 (64)	>0.5
R31A-angiogenin	10	36 (178)	0.0006
R32A-angiogenin	10	47 (51)	<0.0001
R33A-angiogenin	10	21 (57)	>0.5
R66A-angiogenin	10	22 (60)	>0.5
R70A-angiogenin	10	43 (94)	<0.0001

[a] The CAM assay was employed (Knighton et al., 1977; Fett et al., 1985). Each mutant protein sample was tested in at least three separate experiments. Data from multiple experiments were combined (8–20 eggs). [b] The total number of assays performed for each sample, n, is given in parentheses. [c] Significance, p, was calculated from χ^2, based on comparison with water control samples tested simultaneously, which produced a 21% positive response (total 257 eggs).

Table VI: Binding of Placental RNase Inhibitor to Angiogenin Mutants[a]

mutation	$k_a \times 10^{-8}$, M^{-1} s^{-1}	$k_d \times 10^7$, s^{-1}	K_i, fM
none[b,c]	1.8	1.3	0.71
none [Met-(–1)][d,e]	4.0	1.5	0.38
R5A[b]	1.1	39	35
R31A [Met-(–1)][d]	2.8	1.6	0.58
R32A[b]	2.4	7.8	3.3
R33A [Met-(–1)][d]	3.3	2.2	0.66
R66A[b]	1.8	1.8	1.0
R70A[b]	2.4	1.1	0.48

[a] Conditions are 0.1 M Mes, pH 6.0, containing 0.1 M NaCl and 1 mM EDTA, 25 °C. k_a and k_d are the rate constants for association and dissociation, respectively. Each k_a value represents the average of four determinations employing different concentrations of angiogenin mutant; standard deviations ranged from 4 to 18%. K_i values represent k_d/k_a. [b] Protein contains the N-terminal <Glu residue. [c] Kinetic constants are from Lee et al. (1989a,b). [d] Protein was produced in *E. coli* as the Met-(–1) derivative. [e] Values are from Shapiro et al. (1989).

Interaction of Mutant Angiogenins with PRI. The apparent second-order rate constants for association, k_a, of the various arginine mutants with PRI, measured indirectly by competition with RNase A for PRI, do not differ markedly from that for angiogenin (Table VI). The largest change is seen with R5A where k_a is 40% lower. The k_a values of the two mutants prepared as Met-(–1) derivatives are higher than those of the four obtained in the natural <Glu-1 form, consistent with the earlier finding that N-terminal extension of angiogenin by

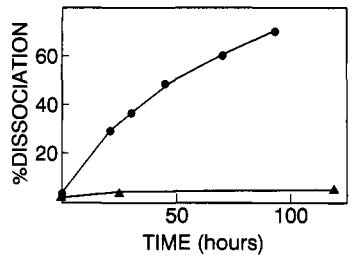

FIGURE 3: Dissociation of the PRI·R5A-angiogenin complex (●). R5A-angiogenin was incubated with 1.5 equiv of PRI for 20 min at 25 °C, followed by addition of 250 equiv of RNase A. Release of free R5A-angiogenin was then measured by HPLC (Lee et al., 1989b). Results obtained simultaneously for the PRI·angiogenin complex (▲) are also shown.

one methionine doubles the rate of association with PRI (Shapiro et al., 1989).

The rate constants for dissociation, k_d, of the mutant protein·PRI complexes were measured by first forming the complex, then adding a large excess of RNase A as scavenger for free PRI, and finally quantitating the angiogenin derivative released after various times by HPLC (Table VI). The complexes with R31A, R33A, R66A, and R70A dissociate at rates similar to that measured with the native protein. The PRI·R32A complex dissociates 6-fold faster. The PRI·R5A complex is considerably less stable (Figure 3), with a k_d of 3.9×10^{-6} s^{-1} compared to 1.3×10^{-7} s^{-1} for the angiogenin complex. These values correspond to half-lives of 2 days and 2 months, respectively.

DISCUSSION

Chemical modification of human angiogenin with arginine reagents rapidly abolishes its enzymatic activity toward polynucleotide substrates (Shapiro et al., 1987). This inactivation, in constrast with that accompanying lysine or histidine modification of angiogenin, cannot be assigned to specific residues based on precedents with RNase A. Although a number of arginine reagents decrease the activity of RNase A (Takahashi, 1968; Yankeelov, 1970; Patthy & Smith, 1975; Iijima et al., 1977; Yamasaki et al., 1980), their effect on this enzyme is less dramatic than that with angiogenin (Shapiro et al., 1987). Moreover, the loss of activity in RNase was attributed to modification of Arg-39 and perhaps Arg-85 (Takahashi, 1968; Patthy & Smith, 1975; Iijima et al., 1977), neither of which has a counterpart in angiogenin: Arg-39 is either deleted or replaced by a proline, depending on the sequence alignment chosen, and lysine substitutes for Arg-85. Indeed, in angiogenin only one of the four arginines of RNase A, i.e., Arg-33, is maintained, and this residue is unreactive in RNase A. Thus, the basis for the response of the two proteins to arginine reagents is completely different.

Owing to the lack of specificity of the reaction, it has been difficult to identify the arginines in angiogenin whose modification abolishes activity: about seven residues react under conditions that inactivate it by 92% (Shapiro et al., 1987). Such lack of specificity is common for modification reactions, but this problem can sometimes be addressed by the use of competitive inhibitors to selectively block the active site. For angiogenin, however, poly(G) is the only inhibitor demonstrated so far to protect against inactivation (Shapiro et al., 1987), and it prevents modification of ~4 residues (unpublished results). Hence, site-directed mutagenesis seemed to be a more promising experimental alternative. In addition to being highly specific, it allows more precise assignment of function to the residue(s) altered since the guanidino moiety is absent rather than blocked through chemical modification with a structurally disruptive, bulky group.

Selection of Residues for Mutation Based on the Dual-Site Model. Six arginines in angiogenin—at positions 5, 31, 32, 33, 66, and 70—were designated for mutation owing to their calculated positions in the three-dimensional structure of the protein (Figure 1; Palmer et al., 1986) relative to the two regions that are required for angiogenic activity. One of these regions is the catalytic site capable of cleaving RNA or some related substrate, and the other is the noncatalytic site thought to bind to a cell-surface receptor (Hallahan et al., 1991, 1992). The former contains His-13, Lys-40, and His-114 analogous to the active site of RNase A; mutation of any of these three abolishes both ribonucleolytic and angiogenic activities (Shapiro et al., 1989; Shapiro & Vallee, 1989). The cell-binding site includes the loops encompassing residues 60–68 (Hallahan et al., 1991) and 107–110 (Hallahan et al., 1992). A number of changes within these loops, including deamidations of Asn-61 and -109 and proteolytic cleavages at Lys-60 and/or Glu-67, markedly diminish angiogenic activity but have no appreciable effect on enzymatic activity or specificity. Although this region does not appear to play a ribonucleolytic role in angiogenin, the corresponding one of RNase A is thought to be involved in substrate binding by forming multiple H-bonds with the purine ring of substrates such as CpA (Richards & Wyckoff, 1973; Wodak et al., 1977).

In the calculated angiogenin structure, four arginines—Arg-5, -31, -32, and -33—are near the enzymatic site. None of them would be expected to form direct contacts either with dinucleotide substrates or with the catalytic histidines or lysine (Richards & Wyckoff, 1973; Wodak et al., 1977). However, any of them could potentially be a component of an extended binding domain for larger substrates, not yet defined crystallographically in RNase. In addition, the guanidino group of Arg-33 is <4 Å from Gln-12 (backbone carbonyl) and Phe-45 (phenyl ring),[3] both residues which may influence activity. Gln-12 is adjacent to the essential His-13 residue and corresponds to Gln-11 of RNase A which may hydrogen-bond to the substrate phosphate group (Wodak et al., 1977). Phe-45 follows Thr-44, which plays an important role in substrate binding (T. P. Curran, personal communication).

Two of the arginines are located within the receptor-binding region. Arg-66 is part of the proposed cell-binding loop 60–68 (Hallahan et al., 1991), and Arg-70 is within hydrogen-bonding distance of both Asn-61 and Glu-67. The former residue has been identified as an essential component of this site (Hallahan et al., 1992).

The balance of the arginines (i.e., Arg-21, -24, -51, -95, -101, -121, and -122) is predicted to be too far away from either site to be critical. The first five are >16 Å from His-13, Lys-40, and His-114 in the enzymatic site and >23 Å from Asn-61 in the cell-binding site. Arg-121 and -122 comprise part of a C-terminal extension which is nonexistent in RNase A, and their positions were not included in the calculated structure. It seems unlikely, however, that they would approach the enzymatic site since the computed C-terminus (Ile-119) is >14 Å from the catalytic residues and the succeeding amino acids should be even further away, in view of the direction of polypeptide chain growth at this point.

Four of the residues selected for mutation are preserved (Arg-5, -33, and -70) or replaced conservatively by lysine

[3] The distance between atoms 1 and 2 $\{=[(x_1 - x_2)^2 + (y_1 - y_2)^2 + (z_1 - z_2)^2]^{1/2}\}$ was calculated from the atomic coordinates of the energy-minimized structure (provided by Dr. K. A. Palmer).

(Arg-31) in the other angiogenins sequenced to date [i.e., bovine, mouse, pig, and rabbit (Bond & Strydom, 1989; Bond & Vallee, 1990b; Bond et al., 1992)]. The other two are not: asparagine and glutamine substitute for Arg-32 in the bovine and porcine proteins, respectively, and asparagine and glycine for Arg-66 in porcine and rabbit angiogenin, respectively. Arg-21 and -101, two additional arginines that are constant in all five angiogenins, were not mutated owing to their distance from both of the critical regions. Alanine substituted for each of the six arginine residues selected in order to assess the magnitude of their contributions to ribonucleolytic and angiogenic activity.

Effect of Mutations on Enzymatic Activity. R33A displays the most striking change in enzymatic activity, a 7-fold decrease. This effect is independent of substrate concentration, indicating that k_{cat} rather than K_m is altered, and is similar for large and small substrates. The mutant protein has the same preference as angiogenin for cytidine over uridine at position N and for adenosine over guanosine at position N′ in NpN′ dinucleotide substrates. These results are consistent with the location of this residue in the three-dimensional structure, and suggest that Arg-33 does not make a significant contribution to substrate binding but may influence the reactivity of catalytic residues. Replacement of Arg-5 also diminishes activity, though less markedly than that of Arg-33. In this instance, the decrease is greater when tRNA is the substrate than when dinucleotides are, perhaps indicating a role for this residue in binding peripheral substrate components. Mutation of the remaining four residues does not affect enzymatic activity appreciably.

Comparison of Chemical Modification and Mutagenesis. The effects of arginine mutations on the ribonucleolytic activity of angiogenin are not comparable to those of chemical modification and underscore the problems inherent in the use of these techniques, particularly the latter, for the attribution of function to structure in proteins. The complete loss of activity toward polynucleotides following treatment with *p*-hydroxyphenylglyoxal, phenylglyoxal, or camphorquinone-10-sulfonic acid (Shapiro et al., 1987) had suggested that arginines in angiogenin are catalytically essential.[4] However, mutation of each of the arginines likely to be near the enzymatic site demonstrates that in fact no one of them is indispensable for activity. Although it is always possible that an arginine that was not mutated plays such a role, their locations argue strongly against it. Arg-21 is particularly unlikely to be critical as it is absent from ARH-III which is as active as angiogenin (Bond & Vallee, 1990a).

How then are the dramatic effects of chemical modification to be understood? The *p*-hydroxyphenylglyoxal reaction typically adds two reagent molecules per guanidino group (Yamasaki et al., 1980). Modification of a functional but noncritical amino acid such as Arg-5 or -33 might therefore have an effect disproportionate to the residue's importance in the native enzyme. Neither of these arginines, however, appears to be involved in the decrease of activity since the reagent inactivates both R5A and R33A at the same rate as angiogenin. Moreover, mutation of Arg-33 to alanine is presumably less disruptive than attachment of the bulky *p*-hydroxyphenylglyoxal groups and yet diminishes activity toward CpA more markedly than does the chemical modification. Modifications of arginines-21, -66, and -70 are also unlikely to underlie the activity loss since ARH-III, R66A, and R70A are again affected by the reagent in the same manner as angiogenin. Two of the mutants, R31A and R32A, however, *are* inactivated more slowly than angiogenin, indicating that modification of Arg-31 and -32 contributes to the activity decrease, albeit indirectly. Simultaneous modifications of both of these residues might then act synergistically and abolish activity toward large substrates altogether. Bovine angiogenin, which lacks these residues, is inactivated much more slowly than the human protein despite the presence of additional arginines at positions 38 and 43 near the catalytic site.

Effect of Mutations on Binding to PRI. Both the enzymatic and angiogenic activities of angiogenin are abolished by PRI (Shapiro & Vallee, 1987), a 50-kDa cytoplasmic protein that binds tightly to all members of the pancreatic RNase superfamily examined thus far (Blackburn et al., 1977; Shapiro et al., 1986a; Shapiro & Vallee, 1991). The dissociation constant for the PRI·angiogenin complex is 0.7 fM (Lee et al., 1989b). Previous mapping of the PRI-binding site on angiogenin has demonstrated that Lys-40 is an important contact point: its mutation to glutamine lowers affinity by a factor of 1300 (Lee & Vallee, 1989a), and even its replacement by arginine has a 100-fold effect (Shapiro et al., 1989). His-114 may likely play a minor role since its substitution by alanine weakens binding by ~3-fold (Shapiro & Vallee, 1989). The fluorescence of Trp-89 increases dramatically upon complex formation, suggesting that this residue is within the angiogenin·PRI contact region (Lee et al., 1989a). Trp-89 does not, however, seem to engage in a specific interaction since neither its oxidation to oxindolylalanine (Lee & Vallee, 1989a) nor its mutation to methionine (E. A. Fox, personal communication) affects K_i significantly. Other amino acids examined—His-13 (Shapiro & Vallee, 1989), Lys-50 and Lys-60 (Lee & Vallee, 1989a), and Asn-61 and Asn-109 (T. W. Hallahan and R. Shapiro, unpublished results)—are not important for binding, nor in fact is the entire segment containing residues 58–70 (Harper & Vallee, 1989).

Ionic interactions make a substantial overall contribution to PRI-binding strength, as indicated by the increase in K_i of more than 6 orders of magnitude as the NaCl concentration is raised from 0.1 to 1.0 M (Lee et al., 1989b). The changes in both association and dissociation rate constants that accompany butanedione treatment of angiogenin (Lee & Vallee, 1989a) suggest that its arginines participate in these interactions. Since mutation of Arg-31, -33, -66, and -70 to Ala does not influence K_i, these can be excluded. However, Arg-32, the immediate neighbor of Arg-31 and -33, does play a role since the alanine mutation increases K_i by about 4-fold. Arg-5 is a major contact point. Its substitution by alanine increases K_i by 50-fold, largely reflecting an accelerated rate of complex dissociation. This residue does not fall within the PRI interaction region defined previously: its inclusion would thus extend this site some distance along the angiogenin surface—Arg-5 is about 15 Å from Lys-40, 9 Å from His-114, and 29 Å from Trp-89 in the calculated structure (Figure 1).

Both of the PRI contact residues identified in this study are replaced nonconservatively in RNase A, which binds 60-fold less avidly than angiogenin (Lee et al., 1989b). Indeed, RNase A contains an alanine at the position corresponding to Arg-5, and this single substitution may therefore account for a large fraction of the difference in binding strength. It should be noted, however, that Arg-5 in human placental RNase is substituted by a posttranslationally modified tryptophan, as yet uncharacterized; nonetheless, this protein binds as tightly to PRI as does angiogenin (Shapiro & Vallee, 1991). If the

[4] Inactivation by these reagents is not due to lysine modification (Shapiro et al., 1987).

modified tryptophan is unable to substitute functionally for the arginine, as seems likely, this would then imply either that the placental RNase contains an additional, compensating contact point absent from angiogenin or that some of the interactions common to both proteins are stronger for the RNase.

Effect of Mutations on Angiogenic Activity. Three of the mutants—R5A, R33A, and R66A—are angiogenically inactive on the CAM at a dose of 10 ng/egg, indicating a decrease of potency >20-fold since 0.5 ng of unmodified angiogenin is sufficient to produce a positive response (Fett et al., 1985). For R5A and R33A, this loss may reflect their decreased ribonucleolytic activities. Although the loss in angiogenic activity appears to be more extensive, the mutations may affect cleavage of some, as yet unidentified, in vivo substrate more dramatically than activity toward the substrates employed in the present work. Alternatively, the cell-binding site on angiogenin may include Arg-5 and -33, although they are relatively far from the residues proposed to be part of this site. Arg-66, the third residue important for angiogenic activity, is in precisely this region of the molecule. Since this amino acid is not conserved in porcine and rabbit angiogenins (Bond et al., 1992), it is unclear whether it plays a direct role; this would then need to be adopted by a different residue in the other proteins. Alternatively, the mutation might perturb critical residues nearby. A more precise understanding of the structural basis for the effects of all three arginine mutations that attenuate angiogenic activity must await X-ray crystallographic or NMR studies.

Summary and Conclusions. Earlier chemical modifications suggested that arginines are important for the ribonucleolytic activity of angiogenin. The present mutagenic study establishes that two arginines, at positions 5 and 33, indeed play a role in activity. Neither residue by itself, however, is crucial, and neither one is implicated in the abolition of enzymatic activity by arginine reagents. Mutations of the four additional arginines, at positions 31, 32, 66, and 70, predicted to lie in the same region of the molecule as the active center, do not significantly impact catalysis. The basis for the effects of chemical modification therefore remains unclear. Inactivation may be a consequence of structural perturbations arising from concurrent modifications of multiple residues which of themselves have no direct functional importance.

In addition to their role in the ribonucleolytic activity of angiogenin, arginines are important both for the binding of angiogenin to PRI and for angiogenic activity. Mutations of Arg-5 and -32 to alanine weaken PRI binding by 50-fold and 4-fold, respectively. Among the numerous angiogenin residues examined to date, the importance of Arg-5 is second only to that of Lys-40. If the contributions of the various contact residues identified (Arg-5, Arg-32, Lys-40, and His-114) are independent and additive, then about 6 orders of magnitude of the K_i value of $\sim 10^{-15}$ M can now be accounted for. The sources of the remainder of the binding strength, whether individual strong contacts, as with Arg-5 and Lys-40, or relatively weak interactions, as with Arg-32, His-114, and perhaps Trp-89, remain to be elucidated.

Finally, arginines appear to play a critical role in angiogenic activity: mutations of three of the six residues examined markedly reduce biological potency. One of these amino acids, Arg-66, lies within the putative cell-binding region. Since R66A is almost fully active enzymatically, this finding lends further support to the dual-site model for the action of angiogenin proposed previously, and provides yet additional evidence of the separability of the enzymatic and angiogenic activities of the protein. The other two arginines, at positions 5 and 33, whose mutations reduce angiogenic activity, are not in this cell-binding site as it is now defined, and the decreased angiogenicity of R5A and R33A may be associated with the changes in ribonucleolytic activity which also accompany the amino acid substitutions.

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Registry No. CpA, 2382-66-3; CpG, 2382-65-2; UpA, 3256-24-4; RI, 39369-21-6; Arg, 74-79-3; angiogenin, 97950-81-7; *p*-hydroxyphenylglyoxal, 24645-80-5.