A Covalent Angiogenin/Ribonuclease Hybrid with a Fourth Disulfide Bond Generated by Regional Mutagenesis†

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ABSTRACT: Human angiogenin is a blood vessel inducing protein whose primary structure displays 33% identity to that of bovine pancreatic ribonuclease A (RNase A). Angiogenin catalyzes limited cleavage of 18S and 28S ribosomal RNA and is several orders of magnitude less potent than RNase A toward conventional substrates. A striking structural difference between angiogenin and RNase is the virtual absence of sequence similarity within the region of RNase that contains the Cys-65–Cys-72 disulfide bond. Indeed, angiogenin lacks this disulfide linkage. The present report describes the use of regional mutagenesis to generate a covalent angiogenin/RNase hybrid protein, ARH-I, where residues 58–70 of angiogenin have been replaced by the corresponding segment of RNase A (residues 59–73). The protein expressed in *Escherichia coli* readily folds at pH 8.5 to form the four expected disulfide bonds. The in vivo angiogenic potency of ARH-I is markedly diminished compared with that of angiogenin when examined using the chick chorioallantoic membrane assay. In contrast, its enzymatic activity is dramatically increased. With high molecular weight wheat germ RNA and tRNA, ARH-I is 660- and 300-fold more active than angiogenin, respectively, while with poly(uridylic acid), poly(cytidylic acid), cytidylyl(3′→5′)adenosine (CpA), and uridylyl(3′→5′)adenosine (UpA) activity is enhanced by about 200-fold. In addition, the specificity of ARH-I toward dinucleoside 3′,5′-phosphates is qualitatively similar to RNase A; while angiogenin prefers cytidylyl(3′→5′)guanosine (CpG) to UpA, both RNase and the hybrid prefer UpA to CpG. ARH-I also displays >10-fold enhanced activity toward rRNA in intact ribosomes, while abolishing the capacity of the ribosome to support cell-free protein synthesis. The enhanced enzymatic properties of ARH-I parallel a 2-fold increase in chemical reactivity of active-site lysine and histidine residues based on rates of chemical modification. The data indicate that introduction of a region of RNase A containing the Cys-65–Cys-72 disulfide bond into angiogenin dramatically increases RNase-like enzymatic activity while reducing its angiogenicity.

The human blood vessel inducing protein angiogenin (Fett et al., 1985; Strydom et al., 1985; Kurachi et al., 1985) is a member of a family of ribonucleolytic enzymes that includes mammalian pancreatic ribonucleases (RNases)[1] and nonsecretory RNases, among others (Bientema et al., 1986, 1988). While the primary structures of these proteins differ in many ways, the three major, well-characterized catalytic residues of the bovine pancreatic enzyme—His-12, Lys-41, and His-119 (Richards & Wyckoff, 1971; Blackburn & Moore, 1982)—are preserved in all of them. Moreover, many of the other active-site residues, including Gln-11, Thr-45, and Asp-121, are also conserved (Strydom et al., 1985; Beintema et al., 1986, 1988). While the pancreatic and nonsecretory ribonucleases noted display similar specific activities toward RNA, the ribonucleolytic action of angiogenin differs markedly. Indeed, angiogenin is several orders of magnitude less potent than RNase A toward conventional substrates and catalyzes the limited cleavage of 18S and 28S rRNA (Shapiro et al., 1986a); in addition, it readily cleaves 18S RNA in intact ribosomes and in the isolated 40S subunit, thereby abolishing the capacity of the ribosome to support cell-free protein synthesis (St. Clair et al., 1987, 1988).

Current efforts aim to identify structural components of angiogenin necessary for angiogenesis. Recent studies have shown that the active-site histidine and lysine residues of angiogenin are required for both angiogenic and enzymatic action (Shapiro et al., 1986a, 1987b, 1988b, 1989). Clearly, additional structural features are critical to angiogenesis since pancreatic RNase contains the corresponding histidine and lysine residues, yet it is not angiogenic. Candidates include individual residues and/or regions of sequence which are unique to angiogenin. One such segment is residues 62–71. Figure 1 shows the alignment of residues 55–75 of angiogenin with the corresponding region of bovine RNase A. Within residues 62–71, there is only one apparent identity (Asn-68), and in angiogenin, two residues are deleted. Moreover, in mammalian pancreatic and nonsecretory RNases, Cys-65 and Cys-72 form a disulfide bond (Smyth et al., 1963; Beintema et al., 1988) which is absent in angiogenin. Crystal structure analysis of RNase S substrate analogue complexes shows that this disulfide bond generates an exposed loop constituting one face of the purine binding site (Richards & Wyckoff, 1973; Wodak et al., 1977). While homology considerations implicate

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[1] Abbreviations: RNase(s), ribonuclease(s); RNase A, bovine pancreatic RNase; HPLC, high-performance liquid chromatography; C18, octadecylsilane; TFA, trifluoroacetic acid; poly(C), poly(cytidylic acid); poly(U), poly(uridylic acid); SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; IPTG, isopropyl thiogalactoside; HSA, human serum albumin; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PRI, placental ribonuclease inhibitor; BMV, brome mosaic virus; UpN and CpN, uridine and cytidine dinucleoside 3′,5′-phosphates where N represents A or G; ARH-I, Met-(−1) angiogenin in which residues 58–70 have been replaced by residues 59–73 in bovine pancreatic RNase A.

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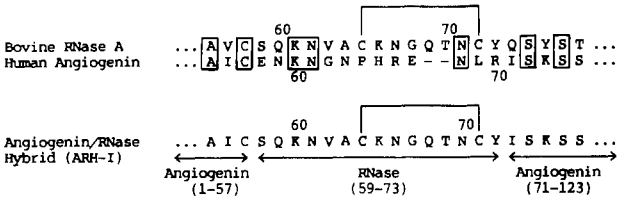

FIGURE 1: Region of the amino acid sequence of angiogenin, bovine RNase A, and the angiogenin/RNase hybrid (ARH-I). Residues in angiogenin, RNase, and ARH-I are designated according to their respective numbering systems (Blackburn & Moore, 1983; Strydom et al., 1985). Conserved residues are boxed. The two amino acid deletion in angiogenin is placed arbitrarily.

the corresponding segment of angiogenin in substrate binding, a preliminary three-dimensional structure of angiogenin (Palmer et al., 1986) suggests that the peptide backbone in this region differs substantially from that of RNase A.

One approach for examining the involvement of such structural elements in angiogenic activity involves the use of regional mutagenesis to generate angiogenin/RNase hybrid proteins, derivatives of angiogenin in which particular regions of primary structure have been replaced with the corresponding segment of RNase. Characterization of such proteins allows segments critical to angiogenin's characteristic activities to be identified. In the present work, we have used regional mutagenesis to prepare a hybrid protein, ARH-I,[1] in which residues 58–70 of angiogenin have been replaced by the corresponding segment of bovine RNase A (residues 59–73) (Figure 1). The functional consequences of including this disulfide-bonded loop structure in angiogenin have been examined not only in terms of angiogenic activity but also with regard to its enzymatic activity and specificity, chemical reactivity of active-site residues, interaction with placental ribonuclease inhibitor, and proteolytic susceptibility. This replacement significantly reduces the angiogenic capacity of angiogenin while dramatically increasing ribonucleolytic activity.

Materials and Methods

Materials. Restriction endonucleases and T4 DNA ligase were from Bethesda Research Laboratories or New England Biolabs. The *Escherichia coli* expression vector pAng2, containing a synthetic angiogenin gene under control of a modified Trp promoter, was prepared as described (Shapiro et al., 1988a). Plasmid pBSKS and *E. coli* strain XL-1 Blue were from Stratagene. RNase substrates were from Sigma Chemical Co. or Calbiochem-Behring. Placental ribonuclease inhibitor (PRI) was purified as described by Blackburn (1979). Bovine RNase A was from Cooper Biochemicals. Oligonucleotides were synthesized on a Milligen instrument and purified by reverse-phase HPLC. DNA sequencing was accomplished by using a Sequenase DNA sequencing kit from United States Biochemicals. Plasmid-derived DNA fragments were purified by using low melting point agarose gel electrophoresis (FMC BioProducts). Unless otherwise noted, angiogenin[2] was expressed in *E. coli* and purified as described (Shapiro et al., 1988a).

Preparation of the ARH-I Expression Plasmid. Fragments of the angiogenin coding sequence were derived from the plasmid pAng2 as shown in Figure 2A. Fragment A (219 bp), encoding Met-(−1) to Ile-56 of angiogenin as well as a

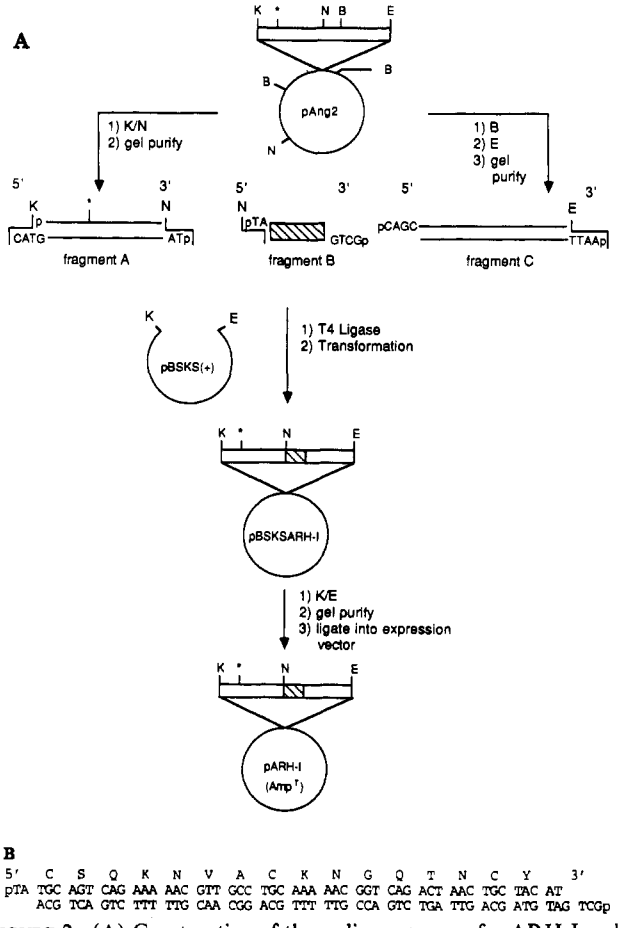

FIGURE 2: (A) Construction of the coding sequence for ARH-I and its insertion into the *E. coli* expression plasmid. Details of the construction are provided in the text, and the sequence of the synthetic angiogenin gene and modified Trp promoter (open rectangle) has been reported (Shapiro et al., 1988a). Restriction endonuclease recognition sites are designated as follows: K, *Kpn*I; N, *Nde*I; B, *Bsp*MI; E, *Eco*RI. Fragment A (219 bp) codes for Met-(−1) to Ile-57 of angiogenin. Fragment B codes for Cys-58 to Tyr-73 of bovine RNase [see (B)] and is indicated as a hatched rectangle. Fragment C (163 bp) codes for Ser-72 to Pro-123 of angiogenin. The asterisk indicates the position of the initiation codon. (B) Synthetic oligonucleotide duplex encoding residues 58–73 of bovine RNase A and containing appropriate 5′ and 3′ termini for ligation with fragments A and B to regenerate Ile-56 and Ile-71 in angiogenin .

portion of the Trp promoter, was obtained by treatment of pAng2 (13 μg) with *Kpn*I and *Nde*I. Similarly, fragment C (163 bp) coding for Ser-72 to Pro-123 in angiogenin was obtained by digestion of pAng2 with *Bsp*MI and *Eco*RI. Two complementary oligonucleotides (fragment B, Figure 2B) coding for residues 58–73 of bovine RNase were synthesized and annealed such that the 5′ and 3′ termini of the duplex would be compatible with the *Nde*I restriction endonuclease recognition site and the 5′ overhang (CAGC) produced by cleavage of pAng2 with *Bsp*MI, respectively [see Shapiro et al. (1988a) for the structure of the synthetic gene]. Once ligated, codons for Ile-56 and Ile-71 in angiogenin, as well as Cys-57, are regenerated. Fragments A (1.2 pmol), B (4.5 pmol), and C (0.8 pmol) were ligated into *Kpn*I/*Eco*RI-digested pBSKS using T4 DNA ligase (10 units, 92-μL total volume) and XL-1 Blue transformants were selected on ampicillin plates containing X-gal and IPTG. Restriction mapping of plasmid DNA from three β-galactosidase negative transformants indicated that two of the clones contained the 430 bp *Kpn*I/*Eco*RI insert expected for ARH-I. This insert was ligated into an expression plasmid derived from pAng2 (Shapiro et al., 1988a) and *E. coli* W3110 transformants

[2] Angiogenin produced in *E. coli* differs from the authentic protein only with respect to its N-terminal residue: Met-(−1) vs pyroglutamic acid-1. This difference has no effect on either ribonucleolytic or angiogenic activity (Shapiro et al., 1988a). For simplicity, bacterially produced Met-(−1) angiogenin will be refered to as angiogenin.

harboring the pARH-I plasmid selected on ampicillin plates.

The *Kpn*I/*Eco*RI fragment of pARH-I containing the presumed hybrid coding sequence was cloned into M13mp18 (RF) and sequenced in its entirety. For this purpose, synthetic oligonucleotides priming second-strand synthesis at the codons for Thr-45 and Gln-93 in angiogenin, in addition to the universal M13 primer, were employed. The sequence obtained was identical with that expected.

Expression and Purification of ARH-I. The levels of expression of six individual colonies of W3110 cells transformed with pARH-I were assessed by immunoblotting using affinity-purified anti-angiogenin as described (Shapiro et al., 1988a). Cells displaying the highest level of expression (~8 mg/L) were used for ARH-I expression.

Large-scale expression and purification were performed as described previously for angiogenin (Shapiro et al., 1988a) except that the lysozyme treatment was for 2 h and there was no sonication. Peak fractions after cation-exchange and reverse-phase HPLC (Shapiro et al., 1988a) were dialyzed against water prior to testing for angiogenic and ribonucleolytic activity.

Structural Characterization of ARH-I. SDS–PAGE was performed as described (Fett et al., 1985). Amino acid analysis was performed using Picotag methodology (Waters Associates) as described (Strydom et al., 1985). For peptide mapping, 100 μg of ARH-I was digested with trypsin (3%) in 10 mM Tris-HCl, pH 8.0, containing 0.35 M NaCl for 24 h at 37 °C, and peptides were purified by C18 HPLC as described (Shapiro et al., 1988a). Peptide compositions were determined by amino acid analysis.

For determination of free cysteine content, ARH-I (40 μg) was treated with 20 mM iodoacetamide in 40 mM Hepes, pH 7.5, containing 160 mM NaCl for 70 min at 37 °C. The protein was purified by C18 HPLC and the extent of carboxymethylation assessed by amino acid analysis.

Enzymatic Assays. All pH values are corrected for the incubation temperature employed. Stock solutions of angiogenin and ARH-I were quantitated by amino acid analysis.

Activity toward tRNA was determined by using a precipitation assay under conditions optimal for angiogenin (Shapiro et al., 1987b). In experiments where the tRNA concentration was varied, all reaction mixtures were adjusted to 3.5 mg/mL tRNA immediately before addition of perchloric acid. Activity toward high molecular weight wheat germ RNA (2 mg/mL) was measured as described by Blank and Dekker (1981). Incubations were performed in 35 mM Tris-HCl, pH 8.1, containing 50 mM sucrose and 150 mM NaCl. Activities toward poly(C) and poly(U) (400 μg/mL) were measured at pH 7.6 by using a modification of the method of Zimmerman and Sandeen (1965) as described (Shapiro et al., 1986a). Activity toward calf liver 18S and 28S rRNA (Pharmacia) was determined by using agarose gel electrophoresis as described (Shapiro et al., 1986a). Activity toward nucleoside 3′,5′-phosphates was measured by HPLC as described (Shapiro et al., 1986a,b).

Inhibition of Cell-Free Protein Synthesis by ARH-I and Analysis of rRNA Fragments. Angiogenin (30 or 70 nM) or ARH-I (0.6–120 nM) was incubated with 33 μL of nuclease-treated rabbit reticulocyte lysate (Promega) for 15 min at 30 °C (final volume 46 μL). One microliter of PRI (11.5 μM) was added, and the mixture was incubated for 10 min at 30 °C to stop the cleavage reaction. In vitro translation was initiated by addition of 1 μL of [^{35}S]methionine (1200 Ci/mmol, 9.6 μM; New England Nuclear), 1 μL of a mixture of all other amino acids at 1 mM, and 1 μL of 0.5 mg/mL BMV RNA, and the mixture was incubated for 60 min at 30 °C. Appropriate controls lacking either BMV RNA or angiogenin were included. Translation was terminated by addition of 2 μL of the reaction mixture to 1 mL of 1 M NaOH containing 1.5% H_2O_2 (10 min, 37 °C) and the extent of radioactivity incorporated into translation products determined by TCA precipitation as described by the supplier.

For isolation of RNA fragments, the translation mixture (40 μL) was diluted with 450 μL of ice-cold 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and 0.5% Nonidet P-40, and 10% SDS (25 μL) was added. RNA fragments were purified by phenol/chloroform extraction and ethanol precipitation. RNA 5′ termini were labeled with 0.34 μM [γ-^{32}P]ATP (3000 Ci/mmol, New England Nuclear) and T4 kinase (0.4 unit/μL) using a kit from Bethesda Research Laboratories (total volume 20 μL). After ethanol precipitation, labeled RNA (1×10^5 cpm) was subjected to 7 M urea/10% polyacrylamide gel electrophoresis (250 V, 2 h). RNA fragments were visualized by autoradiography.

Chemical Modification of ARH-I. Modifications of ARH-I (28 μM) or angiogenin (43 μM) were performed essentially as described (Shapiro et al., 1987b). Modification with 30 mM bromoacetate was performed in 25 mM sodium acetate, pH 5.6, at 37 °C. Reductive methylation was carried out with 2 mM formaldehyde and 10 mM $NaCNBH_3$ in 20 mM Mes, pH 6.0, containing 20 mM NaCl at 25 °C. Modification with 10 mM *p*-hydroxyphenylglyoxal was performed in 25 mM Hepes, pH 8.0, at 25 °C. Reaction mixtures were diluted 60-fold (angiogenin) or 30000-fold (ARH-I) prior to activity determination using tRNA as substrate (2 h, 37 °C). Appropriate standard curves were included in each set of assays. Absorbance readings were corrected for any contribution of the modification reagent.

Interaction of ARH-I with Placental Ribonuclease Inhibitor. Rates for association of PRI with RNase and either angiogenin or ARH-I were measured by using the method of Lee et al. (1988a).

Dissociation of the ARH-I complex was examined by HPLC (Lee et al., 1988b). ARH-I (2 μg at 0.7 μM) was incubated with or without 1.5 equiv of PRI in 100 mM Mes, pH 6.0, containing 100 mM NaCl, 1 mM EDTA, and 120 μM DTT for 20 min at 25 °C, and 250 equiv of RNase A was added as scavenger for free PRI. The quantity of free ARH-I was quantitated by cation-exchange HPLC immediately after addition of RNase A and after 48 h.

Biological Assays. Angiogenic activities of ARH-I and angiogenin were assessed by using the chick embryo chorioallantoic membrane method of Knighton et al. (1977) as described (Fett et al., 1985).

Results

Construction of the ARH-I Hybrid Coding Sequence. Figure 2 shows the strategy for construction of an angiogenin/RNase hybrid protein in which the entire region containing residues 58–70 of angiogenin is replaced by the corresponding segment of bovine RNase A (residues 59–73). The hybrid ARH-I coding sequence was derived from three DNA fragments designated A, B, and C as described under Materials and Methods. The vector pBSKS(+), previously cleaved at unique *Kpn*I and *Eco*RI sites within the β-galactosidase coding sequence, was used to assemble fragments A, B, and C. Since the configuration of compatible 5′ and 3′ termini is unique (Figure 2A), bacterial colonies lacking β-galactosidase activity would be expected to harbor the pBSKS–ARH-I plasmid. Of the three transformants examined, two were found to have a 430 bp *Kpn*I–*Eco*RI plasmid insert, consistent with the size

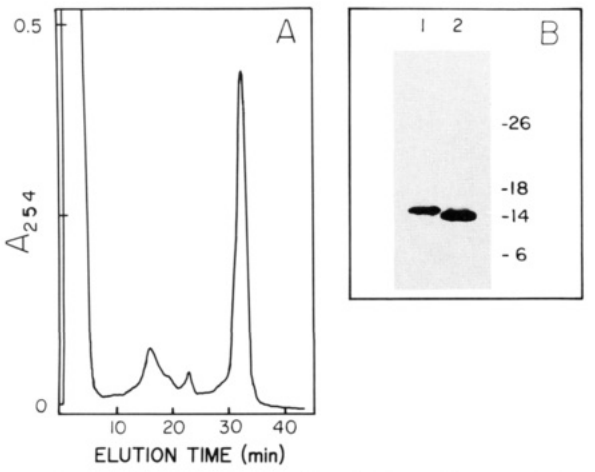

FIGURE 3: (A) Chromatography of partially purified ARH-I on a Mono S cation-exchange column. Solvent A was 10 mM Tris (pH 8.0), and solvent B was solvent A supplemented with 1 M NaCl. Elution was accomplished with a 50-min linear gradient from 15% to 55% solvent B at a flow rate of 0.8 mL/min. (B) SDS–PAGE of 200 ng of ARH-I (lane 1) and angiogenin (lane 2) in a 15% polyacrylamide gel. Protein was visualized by silver staining. Positions of molecular weight markers (Besthesda Research Laboratories) are at the right ($\times 10^{-3}$).

expected for the hybrid coding sequence. The *Kpn*I–*Eco*RI fragment from one of the clones was placed into an *E. coli* expression plasmid containing a modified Trp promoter (Shapiro et al., 1988a) to give pARH-I (Figure 2A). In order to rule out spurious mutations, the ARH-I coding region was sequenced in its entirety. The hybrid gene encodes a protein of 126 amino acids [including Met-(−1)] with residues 58–72 corresponding to residues 59–73 in RNase (Figure 1).

Expression and Purification of ARH-I. The hybrid protein was expressed in *E. coli*, and refolding and disulfide bond formation were achieved by using protocols previously established for angiogenin (Shapiro et al., 1988a). Briefly, partially purified ARH-I was denatured and reduced with 7 M guanidine and 100 mM 2-mercaptoethanol (pH 7.4, 3 h, 37 °C) and the mixture diluted 100-fold (pH 8.5) to allow for refolding (4 °C, 22 h). Cation-exchange HPLC of this material on a Mono S column revealed the presence of one major peak eluting at 33 min (Figure 3A), about 3 min later than angiogenin itself. Peak fractions were purified further by reverse-phase HPLC. The protein obtained is >98% pure as assessed by SDS–PAGE (Figure 3B) and migrates as a single band of $M_r \sim 15000$. The yield of homogeneous ARH-I was 2 mg/L of culture.

Structural Characterization of ARH-I. The amino acid composition of the protein is consistent with the proposed structure (Table I). The hybrid protein contains 7.9 half-cystine residues as assessed by performic acid oxidation to cysteic acid (Strydom et al., 1985) while in parallel analyses angiogenin showed 5.9 residues of cysteic acid.

In order to determine whether any free cysteine residues were present in ARH-I, modification with iodoacetamide was performed (see Materials and Methods). (Carboxymethyl)-cysteine was not detected by analysis of 160 pmol of this material, indicating the absence of free cysteine residues.

Tryptic peptide mapping was carried out in order to establish the pattern of disulfide bond pairing and to confirm the absence of unintentional structural alterations. Peptides were fractionated by HPLC (Figure 4) and their compositions determined by amino acid analysis. The peptides obtained account for all but two residues—Arg-32 and Arg-33—of the protein. The compositions of peptides T-9, T-10, and T-11′

Table I: Amino Acid Compositions of Angiogenin and ARH-I[a]

amino acid	angiogenin	ARH-I	amino acid	angiogenin	ARH-I
Asp	15	14.8 (14)	Tyr	4	4.5 (5)
Glu	10	10.7 (10)	Val	5	5.7 (6)
Ser	9	9.5 (10)	Met	2	1.8 (2)
Gly	8	8.0 (8)	Ile	7	7.5 (7)
His	6	4.8 (5)	Leu	6	5.1 (5)
Arg	13	11.2 (11)	Phe	5	5.2 (5)
Thr	7	8.5 (8)	Lys	7	8.4 (8)
Ala	5	6.3 (6)	Cys[b]	6	7.9 (8)
Pro	8	6.7 (7)			

[a] Relative molar amounts of amino acids are given for ARH-I and are based on HPLC quantitation of acid hydrolysates after derivatization with phenyl isothiocyanate (Strydom et al., 1985). The composition of angiogenin is from the amino acid sequence (Strydom et al., 1985), and the number of residues expected for ARH-I based on the sequence of angiogenin and the replacement made is given in parentheses. Analyses were performed in duplicate on 200–300 pmol of protein. [b] Half-cystine content was determined as cysteic acid after performic acid oxidation (Strydom et al., 1985).

Table II: Amino Acid Compositions of Selected Tryptic Peptides from ARH-I[a]

amino acid	peptide				
	T-5a	T-5b	T-8, T-9[b]	T-10	T-11′
Asp	2.90 (3)	3.14 (3)	5.02 (5)	0.58	2.53 (3)
Glu	1.27 (1)	1.07 (1)	2.18 (2)	1.44 (1)	3.00 (3)
Ser	1.20 (1)	1.06 (1)	2.99 (3)	2.17 (2)	2.09 (2)
Gly	2.00 (1)	1.62 (1)	1.28 (1)	3.05 (3)	1.43 (1)
His	0.37		1.00 (1)	1.00 (1)	0.93 (1)
Arg	0.13		2.84 (3)	1.32 (1)	1.00 (1)
Thr	1.40 (1)	1.22 (1)	3.42 (3)	1.85 (1)	0.24
Ala	1.00 (1)	1.13 (1)	0.16	0.36	2.12 (2)
Pro	0.20	0.12	0.18	3.50 (4)	1.07 (1)
Tyr	0.90 (1)	0.99 (1)	0.98 (1)	1.15 (1)	
Val	1.10 (1)	1.17 (1)	1.18 (1)	0.22	3.53 (4)
Met	0.32		0.82 (1)	0.47	
Ile	1.00 (1)	0.95 (1)	3.13 (3)	0.44	2.02 (2)
Leu	0.23	0.21	0.13	1.78 (2)	1.94 (2)
Phe	0.37		2.11 (2)		1.06 (1)
Lys	1.90 (2)	2.12 (2)	2.06 (2)	1.00 (1)	0.97 (1)
Cys[c]	2.30 (2)	1.90 (2)	2.30 (2)	1.80 (2)	2.04 (2)
pmol anal	125	325	121	20	125

[a] Analyses were carried out as described (Strydom et al., 1985). Relative molar amounts of amino acids are given. The number of residues expected in each peptide (in parentheses) is based on the sequence of angiogenin and the replacement made. Analyses are not corrected for Asp, Glu, Ser, and Gly which are present in some HPLC fractions at this level. Quantities less than 0.1 residue are not indicated. The number of picomoles of peptide analyzed is given at the bottom of the table. [b] In the chromatographic system employed, peptides T-8 and T-9 comigrate, and the composition provided is calculated on the basis of an equimolar mixture of the two peptides contained in the peak HPLC fraction. The composition obtained is in excellent agreement with that expected from the sum of the residues in the individual peptides (in parentheses). [c] Half-cystine residues are determined after performic acid oxidation as described under Materials and Methods.

(Table II) demonstrate that the three disulfide bonds of native angiogenin (Cys-26–Cys-81, Cys-39–Cys-92, and Cys-57–Cys-107) are maintained in ARH-I. As expected, peptide T-11′ from ARH-I (residues 55–60 + residues 104–123) contains two Ser and three Asp residues while the corresponding peptide in angiogenin contains one Ser and four Asp residues. In addition, the compositions of peptides T-1, T-2, T-3b, T-6, T-7, and T-8 are essentially identical with those of angiogenin.

The ARH-I digest does not include peptides containing residues 61–73 in angiogenin—T-4a, T-5, and T-3b [Figure 4, insert; also see Shapiro et al., (1988a)]. However, there are two new peptides, T-5a and T-5b (Figure 4), both with

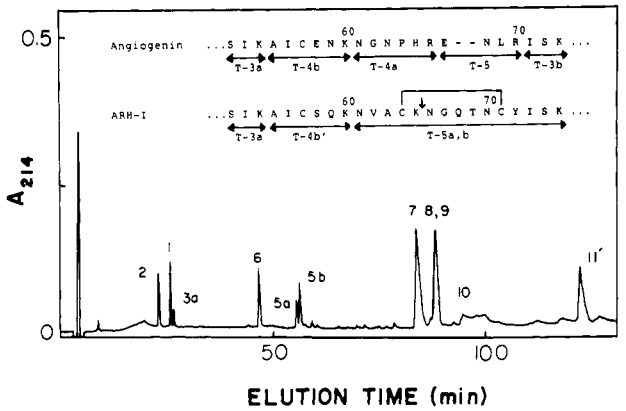

FIGURE 4: Fractionation of tryptic peptides of ARH-I on a Ultrasphere C18 HPLC column. ARH-I was digested with trypsin as described under Materials and Methods. Solvent A was 0.1% TFA, and solvent B was 0.08% TFA in a 3:2:2 (v/v) mixture of 2-propanol, acetonitrile, and water. Elution was accomplished with a 140-min linear gradient from 0% to 50% solvent B at 0.8 mL/min. Peptide designations are from a previous study (Strydom et al., 1985) except for peptides T-4b', T-5a, T-5b, and T-11'. Insert: a portion of the primary structures of angiogenin and ARH-I and the position of relevant tryptic peptides. Peptides T-4b and T-4b' are disulfide bonded through Cys-57 to residues 104–123 of angiogenin to form peptides T-11 and T-11' for angiogenin and ARH-I, respectively [see Strydom et al. (1985) and Table II]. The arrow in the ARH-I sequence shows the position of a potential trypsin cleavage site in peptide T-5 giving rise to two peptides linked by a disulfide bond between Cys-64 and Cys-71 (ARH-I numbering system).

Table III: Angiogenic Activity of ARH-I and Angiogenin[a]

sample	dose (ng)	% positive responses
angiogenin	5	50 (70)[b]
	1	46 (71)
ARH-I	5	30 (60)
	1	31 (67)
	0.1	24 (66)

[a]The chick embryo chorioallantoic membrane assay was employed (Knighton et al., 1977; Fett et al., 1985). Between 10 and 15 eggs were used for each set of assays. Assays for angiogenin and ARH-I were performed simultaneously. For some experiments, angiogenin derived from a mammalian expression system (Kurachi et al., 1988) was employed. The potency of this material is indistinguishable from that of *E. coli* angiogenin. Control samples containing only water produced 14% positive responses (total of 63 eggs). [b]The total number of assays performed for each sample is given in parentheses.

compositions that are indistinguishable from that expected for residues Asn-61 to Lys-75 in ARH-I (Table II; Figure 4, insert). Cysteic acid analysis after performic acid oxidation confirmed the presence of two half-cystine residues in both peptides. The existence of two peptides with essentially identical compositions suggests partial cleavage of the Lys-65–Asn-66 bond, with the resultant peptides linked by a disulfide bond between Cys-64 and Cys-71 (Figure 4, insert).

Biological Activity. In the chick embryo chorioallantoic membrane assay, angiogenin typically produces 50% positive angiogenic responses at 1 ng/egg (Table III). The activity of ARH-I, however, is substantially diminished; at 1 or 5 ng/egg, only ~30% of the responses were positive. It was conceivable that as a result of its increased catalytic activity (see below) ARH-I might display angiogenic activity at somewhat lower doses than does angiogenin. This does not appear to be the case. At 0.1 ng/egg, the percentage of positive responses is only slightly higher than that of blank samples.

Ribonucleolytic Activity of ARH-I. The choice of assays employed allows the assessment of the enzymatic activity of ARH-I in terms of both standard pancreatic RNase-type

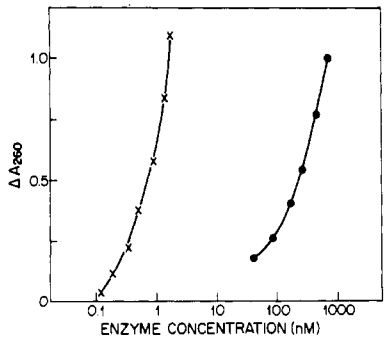

FIGURE 5: Cleavage of tRNA (2 mg/mL) by ARH-I (×) and angiogenin (●). Incubations were performed in 30 mM Hepes/NaCl, pH 6.8, for 2 h at 37 °C. Reactions were terminated by addition of perchloric acid and acid-soluble RNA fragments quantitated by the absorbance at 260 nm (Shapiro et al., 1988a).

activity and specific ribonucleolytic activity of angiogenin. In all cases, the activity of ARH-I is enhanced significantly. When tRNA is the substrate, ARH-I is ~300-fold more active than angiogenin (Figure 5). In this assay, the dependence of ΔA_{260} on angiogenin concentration is markedly nonlinear (Shapiro et al., 1988a), while with ARH-I the formation of acid-soluble products is linear up to ΔA_{260} values of 1.2 (not shown). Thus, the extent of enhancement depends on the ΔA_{260} value used for comparison. For example, with 1 nM ARH-I and 300 nM angiogenin, the same quantity of acid-soluble RNA fragments are produced during the 2-h assay period. Under these conditions, RNase A is ~200-fold more active than ARH-I.

The activity of ARH-I toward wheat germ RNA was measured under conditions optimal for RNase A (pH 8.1, 150 mM NaCl) and found to be 660-fold greater than for angiogenin, but 550-fold less than for RNase A. The hybrid enzyme is 190- and 170-fold more active than angiogenin toward poly(C) and poly(U), respectively, but about 3 orders of magnitude less than RNase A.

Angiogenin catalyzes the limited cleavage of 18S and 28S rRNA as assessed by agarose gel electrophoresis (Figure 6, lanes 2–4) (Shapiro et al., 1986a, 1987a). With 0.6 μM enzyme, polynucleotide products 100–500 nucleotides in length are formed after a 60-min incubation period (lane 4). When the angiogenin concentration is increased by an order of magnitude, these products are still prominent (lane 5). ARH-I (12 nM) also cleaves rRNA in a time-dependent fashion (Figure 6, lanes 6–10). Comparison with the angiogenin time course indicates that ARH-I is ~20-fold more active than angiogenin in this assay. Incubation with 120 nM ARH-I generates polynucleotide products of ~500 bases in length (lane 11), similar to that produced by angiogenin. However, these fragments are completely degraded when the enzyme concentration is increased 10-fold (lane 12).

Activity of ARH-I toward Dinucleoside 3',5'-Phosphates. Four dinucleoside phosphates were used to determine k_{cat}/K_m values (Table IV) for transphosphorylation by ARH-I and angiogenin employing an HPLC method that allows the quantitation of very slow cleavage rates (Shapiro et al., 1986a,b). In all cases, ARH-I is substantially more reactive than angiogenin. Activity toward CpA and UpA is increased ~200-fold, while with CpG and UpG, it is increased 10-fold and 30-fold, respectively. Activities of ARH-I are still 2–4 orders of magnitude lower than those of RNase A (Table IV).

Dependence of Ribonucleolytic Activity on pH and Substrate Concentration. Cleavage of tRNA by ARH-I was assessed over the range of pH from 5.4 to 9.8 as described (Harper & Vallee, 1988a). As with angiogenin, activity is

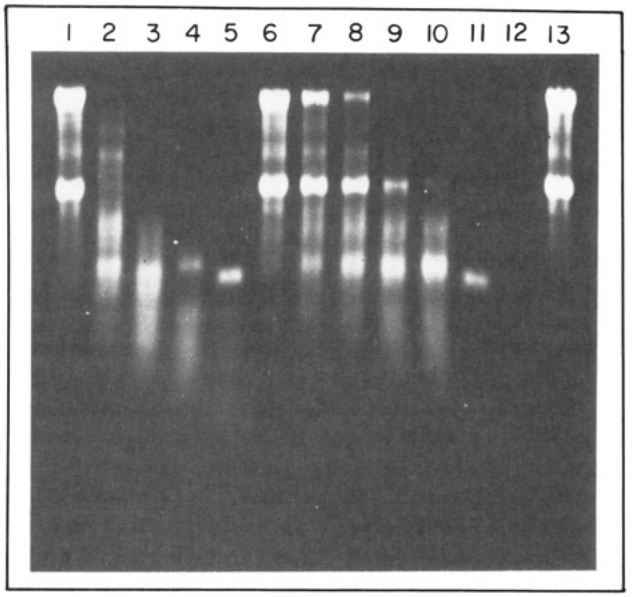

FIGURE 6: Cleavage of 18S and 28S rRNA by angiogenin and ARH-I. rRNA (1.1 mg/mL) was incubated with either angiogenin or ARH-I in 30 mM Hepes/NaCl, pH 6.8, at 37 °C. Reactions were terminated by addition of a 15:5:3 mixture (v/v) of deionized formamide, 37% formaldehyde, and 200 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.0, containing 50 mM sodium acetate and 10 mM EDTA. RNA was separated on a 1.1% agarose–formaldehyde gel and visualized with ethidium bromide. (Lane 1) Control RNA incubated for 60 min; (lanes 2–4) samples containing 0.6 μM angiogenin incubated for 4, 20, and 60 min, respectively; (lane 5) sample containing 6.3 μM angiogenin incubated for 60 min; (lanes 6–10) samples containing 12 nM ARH-I incubated for 4, 10, 20, 30, and 60 min, respectively; (lane 11) sample containing 120 nM ARH-I incubated for 60 min; (lane 12) sample containing 1.2 μM ARH-I incubated for 60 min; (lane 13) same as lane 1.

Table IV: Values of k_{cat}/K_m for Cleavage of Dinucleoside 3′,5′-Phosphates by ARH-I, Angiogenin, and RNase A[a]

substrate	k_{cat}/K_m (M^{-1} s^{-1})		
	ARH-I	angiogenin	RNase A[b]
CpA	2840	12	6000000
UpA	220	1.1	4000000
CpG	50	4.0	510000
UpG	11	0.4	180000

[a] Incubations were performed at 37 °C in 30 mM Mes/NaCl (pH 5.9) containing 0.1 mg/mL HSA, 0.1 mM substrate, and 0.3–5.0 μM enzyme. Aliquots (15–20 μL) were applied at various times to a Waters Radial-PAK C18 column for quantitation of substrate and products. Values for k_{cat}/K_m were calculated by using the expression $k_{cat}/K_m = \ln([S]_0/[S]_t)/t[E]$ where $[S_0]$ and $[S]_t$ are the substrate concentrations at the initial time and time t, respectively, and [E] is the enzyme concentration. [b] Values are from a previous study (Harper et al., 1988) and were determined spectrophotometrically in 30 mM Mes/NaCl, pH 6.0, 25 °C.

optimal near pH 6.8 (not shown). Moreover, the overall shape of the profile is very similar for both enzymes. In this assay, the optimum for RNase A is near pH 8.

ARH-I activity was measured over a range of tRNA concentrations from 0.1 to 3.5 mg/mL in order to assess whether increased activity reflects enhanced substrate binding or turnover. When 0.75 nM ARH-I and 270 nM angiogenin were used, no difference in the substrate concentration dependence was discernible; in both cases, half-maximal activity was observed at ~0.3 mg/mL (not shown). This suggests that enhanced substrate turnover is primarily responsible for the increased activity of ARH-I.

Inhibition of Cell-Free Protein Synthesis by ARH-I. Angiogenin is a potent inactivator of cell-free protein synthesis by virtue of its capacity to selectively cleave the 18S RNA

Table V: Effect of Angiogenin and ARH-I on the Translational Capacity of the Rabbit Reticulocyte Lysate[a]

enzyme	concn (nM)	incorporation of [^{35}S]Met (% inhibition)
angiogenin	30	64
	70	98
ARH-I	0.6	59
	6.0	97
	30	>99
	70	>99
	120	>99

[a] In vitro translation was performed as described in the text. Percent inhibition is based on untreated controls (4.1×10^5 cpm) and control incubations lacking BMV RNA (400 cpm).

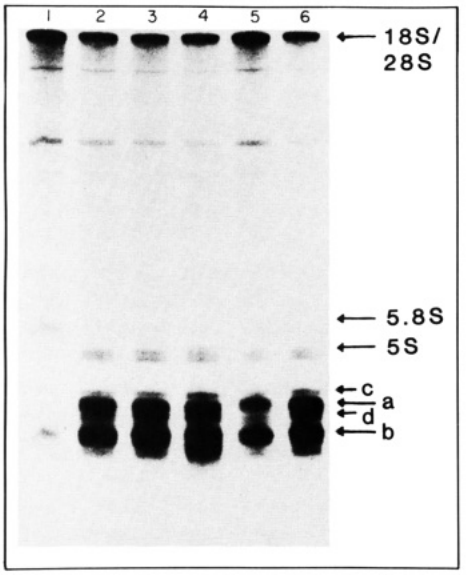

FIGURE 7: Electrophoretic analysis of rabbit reticulocyte rRNA after treatment with angiogenin or ARH-I. 5′ termini were radiolabeled as described in the text. (Lane 1) RNA isolated from lysates incubated in the absence of either angiogenin or ARH-I; (lanes 2 and 3) samples treated with 30 and 70 nM angiogenin, respectively; (lanes 4–6) samples treated with 120, 0.6, and 6.0 nM ARH-I, respectively. Major low molecular weight RNA fragments generated are designated a–d. The positions of 5.8S, 5S, and 18S/28S rRNA are also indicated. After longer exposure periods, fragments migrating slightly faster than 5S rRNA are apparent in all of the treated lanes, and in addition, a fragment migrating between 5.8S and 5S is present in lane 4.

component in the 40S ribosome (St. Clair et al., 1987, 1988). As shown in Table V, ARH-I also is an effective inactivator of protein synthesis in vitro. Pretreatment of reticulocyte lysates with as little as 6 nM ARH-I almost completely abolishes the translation of exogenous viral RNA. Even at 0.6 nM ARH-I, 59% inhibition is observed. With angiogenin, there is 64% inhibition with 30 nM enzyme, and with 70 nM angiogenin, it is complete (Table V). Thus, in this assay, ARH-I appears to be at least 1 order of magnitude more effective than angiogenin.

This enhanced activity may result from an increased rate for the specific RNA cleavage reaction previously characterized for angiogenin (St. Clair et al., 1987, 1988) or, alternatively, from a more general degradation of rRNA characteristic of RNase A (St. Clair et al., 1987). Hence, the effect of ARH-I on the structural integrity of reticulocyte rRNA was compared directly with that of angiogenin by employing urea–polyacrylamide gel electrophoresis (Figure 7). Treatment with 30 nM angiogenin generates several low molecular weight polynucleotides as identified by urea/polyacrylamide gel electrophoresis (lane 2) which are not present

in the RNA from untreated lysates (lane 1). Fragments generated include two prominent bands (designated a and b in Figure 7) as well as two less prominent bonds (designated c and d), all of which migrate faster than 5S RNA. With 70 nM angiogenin (lane 3), the pattern is similar, but band d is somewhat more prominent. Treatment with 6 nM ARH-I (lane 6) results in a pattern of products very similar to that produced by angiogenin. At much higher concentrations of ARH-I (lane 4), the overall pattern of cleavage products is maintained. However, the quantity of small fragments (designated b) is somewhat higher, and an additional fragment migrating slower than 5S RNA is evident after longer periods of autoradiography (not shown). It should be noted that with 70 nM RNase A degradation of the rRNA is much more extensive (St. Clair et al., 1987). Thus, the increased efficiency of ARH-I in inactivation of cell-free protein synthesis is apparently due to an enhanced rate of specific cleavage of reticulocyte rRNA.

Chemical Modification of ARH-I. Modifications of essential histidine, lysine, and arginine residues were carried out as described under Materials and Methods. With 30 mM bromoacetate, inactivation occurs with a $t_{1/2}$ of 25 min, 2-fold faster than with angiogenin. Similarly, reductive methylation of critical amino groups with formaldehyde and $NaCNBH_3$ is 2-fold faster with ARH-I ($t_{1/2}$ = 30 min) than with angiogenin. In contrast, the rate of inactivation of ARH-I with 10 mM *p*-hydroxyphenylglyoxal is indistinguishable from that of angiogenin ($t_{1/2}$ = 16 min). Thus, the reactivity of essential lysine and histidine residues is enhanced, but that of essential arginine residue(s) is not altered.

Interaction of ARH-I with PRI. Placental ribonuclease inhibitor (Blackburn et al., 1977) is an extremely potent inhibitor of both angiogenin and RNase (Shapiro & Vallee, 1987; Lee et al., 1988a,b; Blackburn et al., 1977). Recent data indicate that PRI binds 60-fold more tightly to angiogenin than to RNase A ($K_I = 7 \times 10^{-16}$ M) (Lee et al., 1988a,b).

The relative rates of association of ARH-I and angiogenin with PRI were determined by using a competition assay (Lee et al., 1988a). PRI is added to a mixture containing RNase A and varying amounts of either angiogenin or ARH-I. The degree of partitioning of PRI between the two enzymes is determined by measuring the concentration of free RNase. The results of these assays indicate that the association rate of PRI with ARH-I is 2-fold slower than with angiogenin.

Dissociation of the PRI–angiogenin complex is extremely slow ($t_{1/2}$ = 62 days). In contrast, dissociation of the PRI–RNase A complex is much faster ($t_{1/2}$ = 13 h). Dissociation of the PRI–ARH-I complex was examined by HPLC as described previously for angiogenin (Lee et al., 1988b). After 48 h, less than 3% of ARH-I had dissociated, indicating that the rate of dissociation of the PRI–ARH-I complex is comparable to that of the PRI–angiogenin complex.

Susceptibility of ARH-I to Cleavage by Trypsin. Previously, it was shown that trypsin readily acts upon angiogenin at 25 °C with initial cleavage predominantly at the Lys-60–Asn-61 bond (Harper & Vallee, 1988b). RNase A also contains a Lys-Asn sequence at the corresponding position, but it is not cleaved under the same conditions. This suggests that the Cys-65–Cys-72 disulfide bond may enhance the stability of RNase A toward proteolysis in this region of the protein. Thus, it was of interest to examine the susceptibility of ARH-I to trypsin cleavage.

Angiogenin (12 μM) and ARH-I (22 μM) were individually incubated with 1 μM trypsin at pH 8.0 for 20 h at 25 °C, and the extent of degradation was assessed by HPLC as described in the legend to Figure 4. With angiogenin, the elution pattern of peptides was identical with that observed for the fully digested protein (Shapiro et al., 1988a). There was no evidence of uncleaved angiogenin. In contrast, >80% of ARH-I eluted at the position expected for the intact protein. Thus, the presence of a fourth disulfide bond in angiogenin appears to greatly reduce the susceptibility of that protein to cleavage by trypsin. Further, this indicates that cleavage in this region of angiogenin is a prerequisite for hydrolysis of other susceptible bonds.

Discussion

The angiogenin/RNase homology suggests numerous experimental approaches to explore the mechanism of action of angiogenin. Indeed, recognition of this homology was critical to the discovery of angiogenin's characteristic enzymatic activity (Shapiro et al., 1986a) and its interaction with placental ribonuclease inhibitor (Shapiro & Vallee, 1987; Lee et al., 1988a,b). The vastly different enzymatic and biological activities displayed by angiogenin and pancreatic RNases suggest the use of hybrid proteins—in which particular regions of primary structure in one are replaced by those found in the other—to identify structural elements necessary for biological activity. An advantage of regional mutagenesis over single-site mutagenesis is that it allows the evaluation of the functional contribution of an entire segment of primary structure. After delineation of regions of interest, selected substitutions can then be made in order to more precisely identify functional residues contained in the larger structural element. It is important that the architecture of the protein be maintained after regional mutagenesis. Hence, flexible regions of sequence such as surface loops or "mini-domains", as opposed to components integral to overall structure, are the best candidates for replacement. Previously, we have employed a noncovalent version of this approach to examine the involvement of the N- and C-terminal regions of angiogenin in catalysis (Harper et al., 1988). The availability of recombinant expression systems for angiogenin (Kurachi et al., 1988; Shapiro et al., 1988a) makes it possible to produce covalent angiogenin–RNase hybrid proteins.

All known mammalian pancreatic ribonucleases contain four disulfide bonds; one of these links Cys-65 and Cys-72 (Beintema et al., 1986; Blackburn & Moore, 1982). On the basis of X-ray crystallographic analysis of dinucleotide substrate analogue–RNase S complexes, this disulfide bond generates an exposed, six-residue loop that constitutes one face of the purine binding site (Richards & Wyckoff, 1973; Wodak et al., 1977). Several residues within this loop and nearby are fully conserved in the pancreatic enzymes (Gln-60, Lys-66, Asn-67, and Asn-71) (Beintema et al., 1986). In these crystal structures, the side chains of two residues in this loop—Gln-69 and Asn-71—appear to form hydrogen bonds with N(6) of the bound adenosine group. This arrangement of hydrogen bonds is thought to contribute substantially to the preference of RNase A for NpA versus NpG bonds (Wodak et al., 1977). Theoretical studies of CpA binding to RNase A provide a somewhat different view of enzyme–substrate interactions in this region (Brünger et al., 1985). While Asn-71 ($O^{\delta 1}$) and Asn-67 ($O^{\delta 1}$) both appear to form hydrogen bonds with N(6) of adenosine, the side chain of Gln-69 is somewhat removed from N(6) and may hydrogen bond to N(1) through the $N^{\epsilon 2}$ group. In addition, Lys-66 is near the active site and may hydrogen bond to His-119 and Asp-121 through intervening water molecules (Brünger et al., 1985).

One of the most striking structural differences between angiogenin and RNase A is the virtual absence of sequence

similarity within the region corresponding to this loop (i.e., residues 62–71). Indeed, angiogenin lacks the Cys-65–Cys-72 disulfide bond,[3] and two residues are deleted (Figure 1). While in the alignment shown Asn-68 is conserved, in the computed three-dimensional structure of angiogenin (Palmer et al., 1986) its position differs significantly from that of its counterpart in RNase. Moreover, there is little similarity in the overall structure of this loop. On the other hand, the amino acid sequences of human and bovine angiogenin display a relatively high degree of sequence similarity in this region (M. D. Bond and D. J. Strydom, personal communication). Of 13 residues, 7 are identical, and 3 are replaced conservatively. The two-residue deletion is also a feature of the bovine protein. The similarity of the angiogenins and the dissimilarity with RNases point to this region of angiogenin as being functionally important. In the present study, residues 58–70 of angiogenin have been replaced by the corresponding segment of RNase A (residues 59–73), and the effects of this replacement on angiogenic activity, catalytic specificity and reactivity, inhibition of cell-free protein synthesis, proteolytic susceptibility, and interaction with PRI have all been examined.

The design of the hybrid protein took into account structural considerations that would be expected to optimize refolding of the protein, as well as the availability of convenient restriction enzyme recognition sites in the synthetic angiogenin gene employed (Shapiro et al., 1988a). On the basis of the computed angiogenin structure (Palmer et al., 1986), the segment Ala-Ile-Cys in angiogenin (residues 55–57) occupies a position similar to that found for Ala-Val-Cys in RNase A (residues 56–58). Similarly, there is reasonable correspondence near the segment Ser-Lys-Ser-Ser in angiogenin (residues 72–75) and Ser-Tyr-Ser-Thr in RNase A (Figure 1). Thus, replacement of the region encompassing residues 58–70 in angiogenin by the corresponding segment of RNase, including a two amino acid insertion, would not be expected to substantially alter the conformation of the backbone of angiogenin in the regions flanking Cys-57 and Ser-72. The restriction endonucleases *Nde*I and *Bsp*MI were used to cleave the angiogenin coding sequence near the codons for these two residues, respectively, and the RNase segment was generated by using a synthetic oligonucleotide duplex (Figure 2A,B). The reduced and denatured hybrid protein created by this strategy and obtained by expression in *E. coli* readily folds at pH 8.5 to form the four expected disulfide bonds, suggesting that the overall structure of the protein has been maintained.

The angiogenic activity of ARH-I when assayed on the chicken embryo chorioallantoic membrane (Table III) is markedly diminished compared with angiogenin. At 1 or 5 ng/egg, ARH-I produces only ~30% angiogenic responses while angiogenin produces about 50% positive responses at 1 ng/egg. Thus, the introduction of the disulfide-bonded loop of RNase into angiogenin is detrimental to biological activity.

Examination of the enzymatic reactivity and specificity of angiogenin mutants provides a means to examine the relationship between catalytic and angiogenic activity, while concurrently affording insight into the structural basis for the different activities of angiogenin and RNase. The activity of ARH-I was assessed both in standard RNase assays and with tRNA as substrate. In all cases, the hybrid enzyme is at least 2 orders of magnitude more active than angiogenin. However, it still remains at least 3 orders of magnitude less active than RNase A. ARH-I acts on isolated 18S and 28S rRNA to produce a cleavage product pattern similar to that produced by angiogenin, but at a ~20-fold faster rate (Figure 6). However, these cleavage products are much more susceptible to further degradation by ARH-I than by angiogenin.

The specificities of angiogenin and RNase differ substantially as assessed with dinucleoside 3′,5′-phosphate substrates (Table IV). For RNase, the order of reactivity is CpA > UpA ≫ CpG > UpG. The preference for A versus G at the 3′-position is primarily a result of enhanced substrate turnover (Witzel & Barnard, 1962). Angiogenin also acts most readily on CpA, but prefers CpG to UpA by 4-fold, indicating that C in the 5′-position takes precedence to A in the 3′-position. Given the putative involvement of residues within the disulfide loop of RNase in substrate binding (Richards & Wyckoff, 1973; Wodak et al., 1977), it was conceivable that introduction of this feature into angiogenin could alter not only reactivity toward dinucleotide substrates but specificity as well. This is indeed the case. For the hybrid enzyme, there is a ~200-fold increase in k_{cat}/K_m toward CpA and UpA (Table IV), yet the relative activity toward the two substrates is unchanged. In contrast, a much smaller enhancement is observed with CpG and UpG. Thus, the order of reactivity of ARH-I is CpA ≫ UpA > CpG > UpG, qualitatively similar to that of RNase A.

Previous characterization of angiogenin's enzymatic activity has employed both intact and dissociated ribosomes as substrate (St. Clair et al., 1987, 1988). Angiogenin cleaves the 18S RNA component of the 40S subunit in a manner that abolishes the translational capacity of the ribosome, while causing only limited RNA cleavage (St. Clair et al., 1988). RNase A is half as potent as angiogenin in this system, and extensive degradation of both 18S and 28S RNA occurs during ribosomal inactivation (St. Clair et al., 1987, 1988). Thus, this system is useful for comparison of the specificity and reactivity of ARH-I and angiogenin.

ARH-I readily abolishes the capacity of reticulocyte ribosomes to support protein synthesis (Table V). On the basis of the concentration dependence for inhibition, ARH-I is at least an order of magnitude more potent than angiogenin. Since RNase A is somewhat less active than angiogenin, these results suggest that ARH-I maintains the cleavage specificity of angiogenin toward intact ribosomes but acts with an enhanced rate. This view is supported by electrophoretic analysis of RNA fragments generated by treatment with ARH-I (Figure 7). At ARH-I concentrations sufficient to inhibit protein synthesis by 97% (i.e., 6 nM), the pattern of RNA fragments formed is indistinguishable from that produced by 70 nM angiogenin (96% inhibition). Only with much higher concentrations of ARH-I are additional cleavage fragments evident. These data suggest that ARH-I causes ribosomal inactivation in a manner closely similar to that of angiogenin.

The chemical basis for the enhanced ribonucleolytic activity of ARH-I is not known at present. The major alterations in catalysis with RNA appear to involve substrate turnover; there is no change in concentration dependence for tRNA cleavage when compared with angiogenin. In addition, there is no change in pH optimum with this substrate. Chemical and site-specific mutagenic studies have shown that angiogenin, like RNase A, utilizes active-site histidine and lysine residues in catalysis (Shapiro et al., 1987b, 1988b, 1989; R. Shapiro, private communication). However, the reactivity of these residues toward various modification reagents is 3–10-fold lower in angiogenin than in RNase A. A parallel is seen in the lower catalytic activity of angiogenin toward typical

[3] While turtle pancreatic RNase also lacks this disulfide bond (Beintema et al., 1985), its structure in this region differs markedly from angiogenin.

pancreatic RNase substrates, suggesting that the increased RNase activity of ARH-I could be due, in part, to an enhanced reactivity of active-site residues. Therefore, chemical modification was used to test this hypothesis. The rates of inactivation of ARH-I with bromoacetate and with formaldehyde/$NaCNBH_3$ are both 2-fold faster than with angiogenin, indicating that the active-site histidine and lysine residues are more reactive in the hybrid. Indeed, rates of modification are intermediate between those of angiogenin and RNase A (Shapiro et al., 1987b), consistent with the enhanced catalytic activity of the hybrid.

Angiogenin is rapidly and completely inactivated by reagents selective for arginine residues (Shapiro et al., 1987b), suggesting the involvement of one or more arginine residues in ribonucleolytic activity. On the basis of homology considerations, Arg-66 and Arg-70 are expected to be near the putative purine binding site in angiogenin and, thus, could be possible candidates. However, neither of them is present in ARH-I, and still its enzymatic activity is abolished by *p*-hydroxyphenylglyoxal at a rate indistinguishable from that of angiogenin. On this basis, Arg-66 and Arg-70 do not appear to be the residues whose modification results in inactivation.

The dramatic differences between the enzymatic activities of angiogenin and RNase potentially provide important clues regarding the mechanism by which angiogenin induces neovascularization. Available evidence points to the critical role of angiogenin's catalytic residues in biological activity. Placental ribonuclease inhibitor binds tightly ($K_i = 7 \times 10^{-16}$ M) to the active site of angiogenin (Lee et al., 1988a,b; F. Lee, personal communication), thereby abolishing angiogenic activity (Shapiro & Vallee, 1987). In addition, bromoacetate modification of His-13 and His-114 (Shapiro et al., 1986a, 1988b), as well as replacement of Lys-40 by glutamine (Shapiro et al., 1989), markedly reduces angiogenic potency. Moreover, histidine modification diminishes angiogenin's capacity to induce the release of second messengers (e.g., diacylglycerol) in endothelial cells (Bicknell & Vallee, 1988).[4] In all cases, there is a concurrent loss of enzymatic activity upon modification. This suggests a relationship between catalysis and induction of blood vessel growth and points to RNA as a potential target for angiogenin in vivo. This view is consistent with the recent finding that replacement of Asp-116 in angiogenin by histidine enhances both the angiogenic potency and the *specific* ribonucleolytic activity by more than an order of magnitude (Harper & Vallee, 1988a). Overall, these data indicate that maintenance of the catalytic features characteristic of the action of angiogenin is critical to its biological function. However, given that the activity of ARH-I toward RNA in intact ribosomes is increased while its angiogenic activity is diminished, it would appear that the inhibition of protein synthesis is not absolutely linked to angiogenesis.

Most probably, angiogenin serves as a regulatory protein. Since its physiological target has not yet been identified, however, it is not clear whether the attenuated biological activity of ARH-I is a direct result of altered catalytic activity. It is plausible that a predominant feature of angiogenin's mode of action involves *binding* to cellular targets, as opposed to actual catalysis. If this is the case, structural alteration of critical components in angiogenin's active center could interfere with this process.

[4] Preliminary results indicate that ARH-I shows a markedly decreased capacity to induce the release of second messengers in cultured endothelial cells (R. Bicknell and B. L. Vallee, unpublished data).

Acknowledgments

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Registry No. CpA, 2382-66-3; UpA, 3256-24-4; CpG, 2382-65-2; UpG, 3474-04-2.

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Purification and Characterization of Tissue Plasminogen Activator Kringle-2 Domain Expressed in *Escherichia coli*

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ABSTRACT: We have expressed the 174–263 fragment (kringle-2 domain) of human tissue-type plasminogen activator (t-PA) in *Escherichia coli* by secretion into the periplasmic space using the alkaline phosphatase promoter and stII enterotoxin signal sequence. A large portion of the secreted protein is associated with an insoluble cellular fraction. This material can be solubilized by extraction with denaturant and reducing agent and then recovered in active form by refolding in the presence of reduced and oxidized glutathione. Kringle-2 is then easily purified by affinity chromatography on lysine-Sepharose followed by cation-exchange chromatography. The isolated protein has an amino acid composition and N-terminal sequence as expected for the 174–263 fragment of t-PA, indicating that the signal peptide has been properly removed. Circular dichroic spectra suggest that the protein is folded similar to the kringle-4 domain of plasminogen [Castellino et al. (1986) *Arch. Biochem. Biophys. 247*, 312–320]. Equilibrium dialysis experiments indicate a single binding site on kringle-2 for L-lysine having a K_D of 100 μM. Using a method based on elution of kringle from lysine-Separose with ω-aminocarboxylic acids [Winn et al. (1980) *Eur. J. Biochem. 104*, 579–586], we have shown the lysine binding site of t-PA kringle-2 to have a preference for a ligand with 8.8-Å separation between amine and carboxylate functions. Charge interactions with the ϵ-amino group of L-lysine are important in binding since the affinities for N^{ϵ}-acetyl-L-lysine, L-arginine, and γ-guanidinobutyric acid are decreased >2000-fold, 200-fold, and 12-fold, respectively, relative to the affinity for L-lysine. Modification of the ligand α-carboxylate by methylation or peptide bond formation produces only 3- and 7-fold decreases in affinity, respectively, suggesting a less significant role in ligand binding of electrostatic interaction with the ligand α-carboxylate.

Kringles are small (ca. 80 residues) protein domains which have a characteristic three disulfide bonded structure that was first observed in prothrombin (Magnusson et al., 1975). On the basis of sequence homology to prothrombin, kringles were proposed to occur singly in urokinase (Steffens et al., 1982) and factor XII (McMullen & Fujikawa, 1985), twice in tissue-type plasminogen activator (t-PA)[1] (Pennica et al., 1983), and 5 times in plasminogen (Sottrup-Jensen et al., 1978). These domains appear to be independent folding units (Castellino et al., 1981; Trexler & Patthy, 1983; Novokhatny et al., 1984), but a general functional role for kringles is not known. The plasminogen kringles have been extensively studied and serve as prototypes of this domain. One or more of the kringle domains of plasminogen (Thorsen, 1975; Thorsen et al., 1981) are involved in the binding of this protein to fibrin. Plasminogen kringles-1 and -4 have a binding site for L-lysine with kringle-1 having the greater affinity (Vâli & Patthy, 1982; Lerch et al., 1980). Affinity chromatography on immobilized lysine is often employed in the purification of plasminogen (Deutsch & Mertz, 1970). Kringle domains are presumed to interact with fibrin by binding to an exposed lysine side chain. The role of the lysine binding site in fibrin binding is supported by the observation that analogues of lysine such as 6-aminohexanoic acid are potent antifibrinolytic agents (Okamoto et al., 1959).

Human t-PA is thought to be a multidomain protein having a domain homologous to fibronectin type I "finger", an epidermal growth factor-like domain, two kringle domains, and a C-terminal proteolytic domain homologous to the trypsin family of serine proteases (Pennica et al., 1983). Studies of t-PA domain deletion mutants suggest that the kringle-2 domain has a lysine binding site (van Zonneveld et al., 1986a). Both the kringle-2 and the fibronectin type I domain appear to be required for high-affinity binding of t-PA to fibrin (van Zonneveld et al., 1986b; Verheijen et al., 1986). In order to characterize the lysine/fibrin binding site of t-PA kringle-2, we have used recombinant DNA technology to express the

[1] Abbreviations: t-PA, tissue-type plasminogen activator; Gdn-HCl, guanidine hydrochloride; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

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