Mutagenesis of Residues Flanking Lys-40 Enhances the Enzymatic Activity and Reduces the Angiogenic Potency of Angiogenin†

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ABSTRACT: The primary structure of the blood vessel inducing protein angiogenin is 35% identical with that of pancreatic ribonuclease (RNase) and contains counterparts for the critical RNase active-site residues His-12, Lys-41, and His-119. Although angiogenin is a ribonucleolytic enzyme, its activity toward conventional substrates is lower than that of pancreatic RNase by several orders of magnitude. Comparison of the amino acid sequences of RNase and angiogenin reveals several striking differences in the region flanking the active-site lysine, including a deletion and a transposition of aspartic acid and proline residues. In order to examine how these sequence changes alter the functional properties of angiogenin, an angiogenin/RNase hybrid protein (ARH-II), in which residues 38–41 of angiogenin (Pro-Cys-Lys-Asp) have been replaced by the corresponding segment of bovine pancreatic RNase (Asp-Arg-Cys-Lys-Pro), was prepared by regional mutagenesis. Compared to angiogenin, ARH-II has markedly diminished angiogenic activity on the chick embryo chorioallantoic membrane but 5–75-fold greater enzymatic activity toward a variety of polynucleotide and dinucleotide substrates. In addition, the specificity of ARH-II toward dinucleotide substrates differs from that of angiogenin and is qualitatively similar to that of pancreatic RNase. Thus, non-active-site residues near Lys-40 in angiogenin appear to play a significant role in determining enzymatic specificity and reactivity as well as angiogenic potency. An additional angiogenin/RNase hybrid protein (ARH-IV), in which residues 59–71 of ARH-II have been replaced by the corresponding segment of pancreatic RNase, was also prepared. Previously, it was shown that replacing this region in angiogenin to produce the hybrid ARH-I increases activity toward conventional RNase substrates by more than 100-fold [Harper, J. W., & Vallee, B. L. (1989) *Biochemistry 28*, 1875–1884]. The enzymatic activity of ARH-IV is enhanced beyond that observed with either ARH-I or ARH-II; the magnitude of the increase, however, suggests that the effects of these two regional replacements are not entirely independent.

Human angiogenin is a basic protein of 123 amino acids that induces neovascularization (Fett et al., 1985) and exerts a number of effects on cultured endothelial (Bicknell & Vallee, 1988, 1989) and smooth muscle (Moore & Riordan, 1990) cells, including the activation of second-messenger pathways regulated by phospholipases C and A_2. A salient characteristic of angiogenin is its homology to the pancreatic ribonucleases (RNases)[1] (Strydom et al., 1985; Kurachi et al., 1985). Forty-one residues are conserved between angiogenin and RNase A, including virtually all of the residues thought to be important for catalysis and substrate binding. Despite these similarities, the ribonucleolytic activity of angiogenin differs both in specificity and in magnitude from that of RNase A (Shapiro et al., 1986); moreover, RNase A is not angiogenic.

A preliminary three-dimensional structure for angiogenin, computed by energy minimization procedures (Palmer et al., 1986), contains no obvious features that would account for angiogenin's unusual enzymatic activity. The primary catalytic residues are located at positions similar to those found in RNase A, although the precise spatial relations remain uncertain. In addition, a number of putative hydrogen bonds and hydrophobic interactions found in RNase A are maintained in angiogenin.

An alternative approach for identifying structural features that contribute to angiogenin's characteristic enzymatic and biological activities involves the generation of angiogenin/RNase A hybrid proteins by regional mutagenesis (Harper & Vallee, 1989). In these hybrids, particular segments of angiogenin are replaced by the corresponding amino acids of RNase. The properties of two such hybrids, containing RNase segments that differ markedly from their angiogenin counterparts, have been reported previously. One of these, ARH-I, in which angiogenin residues 58–70 are replaced by RNase A residues 59–73, has dramatically increased RNase activity but lacks angiogenic activity (Harper & Vallee, 1989). With the second hybrid, ARH-III, containing RNase A residues

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[1] Abbreviations: RNases(s), ribonuclease(s); RNase A, bovine pancreatic RNase A; ARH-I, Met-(−1) angiogenin in which residues 58–70 have been replaced by residues 59–73 from RNase A; ARH-II, Met-(−1) angiogenin in which residues 38–41 have been replaced by residues 38–42 from RNase A; ARH-IV, Met-(−1) angiogenin in which residues 38–41 and 58–70 have been replaced by residues 38–42 and 59–73 from RNase A, respectively; HPLC, high-performance liquid chromatography; C18, octadecylsilane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; poly(C), poly(cytidylic acid); poly(U), poly(uridylic acid); TFA, trifluoroacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; HSA, human serum albumin; UpN and CpN, uridine and cytidine dinucleoside 3′,5′-phosphates where N represents adenosine or guanosine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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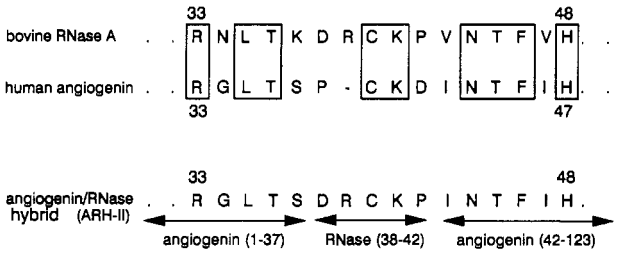

FIGURE 1: Segment of the amino acid sequences of angiogenin, RNase A, and the angiogenin/RNase hybrid (ARH-II). Residues are designated according to their respective numbering systems (Richards & Wyckoff, 1971; Strydom et al., 1985). Conserved residues are boxed.

7–21, angiogenic potency is actually increased, but enzymatic activity toward most substrates is unchanged (Bond & Vallee, 1990).

An additional segment of angiogenin that differs strikingly from RNase A is that encompassing the active-site residue Lys-40. As shown in Figure 1, the major changes are a transposition of aspartic acid and proline residues and a single-residue deletion in angiogenin (or insertion in RNase). Although these changes do not directly involve residues considered to be critical for the catalytic activity of RNase (Richards & Wyckoff, 1971; Brünger et al., 1985), they should alter the backbone structure in this region and may consequently affect the orientation of residues that are critical. Thus, while Lys-40 in angiogenin may occupy a position similar to that found for Lys-41 in RNase A, the precise location of the side chain with respect to the other active-site components could be greatly influenced by the secondary structure imposed by the flanking residues.

In the present work, two angiogenin/RNase hybrid proteins have been constructed in order to examine the extent to which non-active-site residues near Lys-40 in angiogenin contribute to enzymatic specificity and reactivity as well as angiogenic activity. In the hybrid ARH-II, a four-residue segment containing the active-site lysine has been replaced by the corresponding segment of RNase A (Figure 1). This results in the insertion of an arginine (corresponding to Arg-39 of RNase A) and the transposition of aspartic acid and proline residues. The lysine and disulfide-bonded half-cystine residue of angiogenin, however, are retained. ARH-II displays greatly increased enzymatic activity and altered specificity, accompanied by a substantial decrease in angiogenic activity. In addition, we have prepared a double-regional mutant, ARH-IV, which contains the RNase segments of both ARH-I and ARH-II; enzymatic activity is increased further.

Materials and Methods

Materials. Recombinant angiogenin[2] was prepared by using an *Escherichia coli* expression system as described (Shapiro et al., 1988). The plasmid pARH-I, used for expression of the angiogenin/RNase A hybrid ARH-I, is from a previous study (Harper & Vallee, 1989). Oligonucleotides were synthesized with Milligen equipment and purified by reversed-phase HPLC. The sources of other materials are indicated elsewhere (Shapiro et al., 1988; Harper & Vallee, 1989).

Preparation of the ARH-II Coding Sequence. Figure 2A shows the strategy for preparation of the ARH-II coding

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

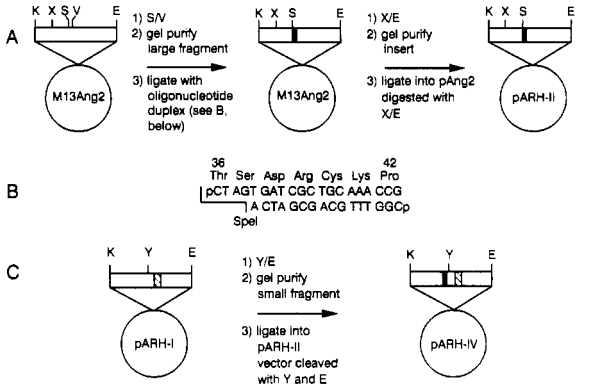

FIGURE 2: Construction of pARH-II and pARH-IV expression plasmids. Details are provided in the text. The sequences of the synthetic angiogenin gene and modified Trp promoter (open rectangle) and the ARH-I coding sequence in pARH-I have been reported (Shapiro et al., 1988; Harper & Vallee, 1989). Restriction endonuclease recognition sites are designated as follows: K, *Kpn*I; X, *Xho*I; S, *Spe*I; V, *Eco*RV; Y, *Sty*I; E, *Eco*RI. (A) Construction of pARH-II. The solid rectangle represents sequences shown in part B. (B) Synthetic oligonucleotide duplex encoding residues 36 and 37 of angiogenin and residues 38–42 of RNase A. (C) Construction of the ARH-IV expression plasmid. In pARH-I, sequences encoding angiogenin residues 58–70 have been replaced by sequences encoding residues 59–73 in RNase A (hatched rectangle).

sequence in which residues 38–41 (Pro-Cys-Lys-Asp) of angiogenin have been replaced by the corresponding segment of RNase A (Asp-Arg-Cys-Lys-Pro) (Figure 1). The M13Ang2 construct (Shapiro et al., 1988) was employed because both the *Spe*I and *Eco*RV recognition sites, which flank sequences encoding Thr-36 through Asp-41, are unique to the angiogenin coding sequence. Double-stranded M13Ang2 cleaved with these two restriction endonucleases was ligated with a synthetic oligonucleotide duplex (Figure 2B) encoding the sequence Thr-Ser-Asp-Arg-Cys-Lys-Pro. After transformation of JM101 cells by the ligated product, recombinant clones were identified by sequencing single-stranded phage DNA. The hybrid coding sequence was then placed into an *E. coli* expression plasmid (Shapiro et al., 1988) as shown in Figure 2A.

Preparation of the ARH-IV Coding Sequence. The construction of the plasmid for expression of ARH-IV is shown in Figure 2C. pARH-I (Harper & Vallee, 1989) was cleaved with *Sty*I and *Eco*RI and the 237-bp fragment encoding His-47 to Pro-123 isolated. Similarly, pARH-II was digested with *Sty*I and *Eco*RI and the plasmid fragment encoding residues 1–46 of the hybrid protein isolated. These two fragments were ligated to generate the final ARH-IV expression plasmid, which was then used to transform W3110 cells for expression. The ARH-IV coding region of the plasmid was sequenced in its entirety to rule out spurious mutations.

Expression, Purification, and Structural Characterization of ARH-II and ARH-IV. Levels of expression of eight individual colonies of W3110 cells that harbored the pARH-II plasmid and eight that harbored pARH-IV were assessed by immunoblotting with affinity-purified anti-angiogenin antibodies (Shapiro et al., 1988), and the highest producers were selected. Expression and purification of each hybrid protein were performed as described (Shapiro et al., 1988) except that indole-3-acrylic acid was added when the A_{600} value was 0.7. After the final C18 HPLC purification step, peak fractions were dialyzed against water. For peptide mapping, ARH-II (40 μg) and ARH-IV (45 μg) were each digested with 3 μg of trypsin (200-μL total volume, 24 h, 37 °C), and the peptides were fractionated on an Ultrasphere C18 column (Beckman Instruments) as described (Shapiro et al., 1988). Amino acid

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

amino acid	angiogenin	ARH-II	ARH-IV	amino acid	angiogenin	ARH-II	ARH-IV
Asx	15	15.6 (15)	13.9 (14)	Pro	8	8.1 (8)	7.1 (7)
Glx	10	10.6 (10)	9.7 (10)	Tyr	4	3.8 (4)	4.6 (5)
Ser	9	8.2 (9)	8.3 (10)	Val	5	4.2 (5)	5.4 (6)
Gly	8	8.5 (8)	8.1 (8)	Met	2	2.0 (2)	2.1 (2)
His	6	5.7 (6)	4.7 (5)	Ile	7	6.7 (7)	6.7 (7)
Arg	13	14.4 (14)	12.0 (12)	Leu	6	6.1 (6)	5.1 (5)
Thr	7	7.1 (7)	7.6 (8)	Phe	5	5.1 (5)	5.0 (5)
Ala	5	5.3 (5)	6.1 (6)	Lys	7	7.2 (7)	8.2 (8)

[a] Relative molar amounts of amino acids are given for ARH-II and ARH-IV and are based on HPLC quantitation of acid hydrolysates after derivatization with phenyl isothiocyanate (Bidlingmeyer et al., 1984). The composition of angiogenin is from the amino acid sequence (Strydom et al., 1985). Analyses were performed in triplicate on 60–100 pmol of protein. The number of residues expected for ARH-II and ARH-IV is given in parentheses.

analysis was accomplished by Picotag methodology (Waters Associates). SDS–PAGE was performed with 15% separating gels in combination with 5% stacking gels.

Enzymatic Assays. Concentrations of angiogenin derivatives were determined by amino acid analysis. Activities toward tRNA, wheat germ RNA, dinucleoside 3′,5′-phosphates, poly(C), and poly(U) were measured by procedures detailed elsewhere (Shapiro et al., 1986a,b; Harper & Vallee, 1989). The pH dependence of ARH-II activity toward tRNA was examined as described previously (Harper & Vallee, 1988).

Angiogenesis Assays. Assays were performed on the chick embryo chorioallantoic membrane by the method of Knighton et al. (1977) as described (Fett et al., 1985).

Chemical Modification. Modifications of ARH-II (4.2 μM) and angiogenin (6.2–10 μM) were performed essentially as described (Shapiro et al., 1987) except that the incubation temperature was 37 °C. Details are provided in the legend to Table IV. Reaction mixtures were diluted 15–30-fold (angiogenin) or 150-fold (ARH-II) at the indicated times, and enzymatic activity was measured with tRNA as substrate (4 h, 37 °C). Appropriate standard curves were included in each set of assays. Absorbance readings were corrected for any contribution of the modification reagent.

Results

Purification and Structural Characterization of ARH-II. ARH-II was expressed in *E. coli* under the control of a modified Trp promoter as described previously for angiogenin (Shapiro et al., 1988). The protein, obtained as inclusion bodies, was denatured and reduced with guanidine and 2-mercaptoethanol, renatured in the presence of air following dilution, and subjected to cation-exchange and reversed-phase HPLC. The yield was 120 μg/L of culture, and the final preparation was >99% homogeneous as judged by SDS–PAGE. The amino acid composition is in excellent agreement with that expected (Table I): the only net effect of the amino acid replacements in ARH-II is to increase the arginine content by one (see Figure 1).

The results of tryptic peptide mapping (Figure 3) suggest that there were no unintentional structural alterations and that the three disulfide bonds had formed properly. The map differs from that obtained for angiogenin in that peptides 8 and 10 are missing and two new peptides, 10a and 14, have been formed. Peptides 8 and 10 in angiogenin represent residues 41–51 and the disulfide-linked residues 34–40 plus 83–95, respectively (Strydom et al., 1985). In the hybrid, a new tryptic cleavage site has been introduced at Arg-39, and the cleavage site at Lys-40 in angiogenin (Lys-41 in the hybrid) has been eliminated due to the placement of a proline on its C-terminal side (Figure 1). The amino acid composition of peptide 10a (Table II) indicates that it contains the disulfide-linked residues 40–52 plus 84–96. Peptide 14 (Table II) then accounts for residues 34–39. The compositions of the remaining peptides T-1, T-2, T-3a, T-4a, T-5, T-6, T-7, T-9, and T-11 (not shown) are consistent with the proposed structure. In total, these peptides account for all but two residues of ARH-II: Arg-32 and Arg-33.

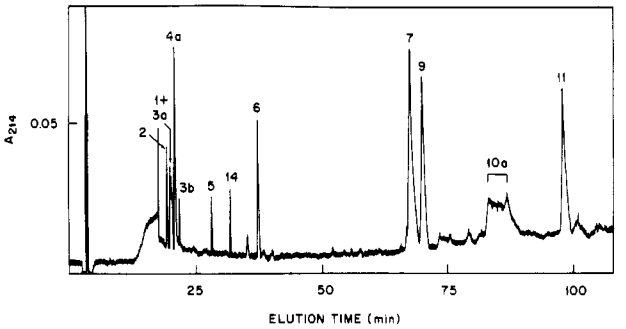

FIGURE 3: Fractionation of tryptic peptides of ARH-II on a Ultrasphere C18 HPLC column. Digestion was performed as described under Materials and Methods. Solvent A was 0.1% TFA, and solvent B was 0.08% TFA in a 3:2:2 (v/v) mixture of 2-propanol, acetonitrile, and water. Elution was accomplished with 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 the new peptides T-10a and T-14.

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

amino acid	T-10a	T-14	amino acid	T-10a	T-14
Asx	2.20 (2)	1.02 (1)	Pro	3.55 (4)	0.19
Glx	1.70 (1)	0.48	Tyr	1.32 (1)	0.11
Ser	1.75 (1)	1.15 (1)	Val	0.32	0.20
Gly	3.80 (3)	1.30 (1)	Met	0.91 (1)	
His	1.75 (2)		Ile	1.70 (2)	0.13
Arg	1.80 (2)	1.00 (1)	Leu	1.34 (1)	0.96 (1)
Thr	1.53 (1)	1.05 (1)	Phe	1.21 (1)	0.16
Ala	0.50	0.21	Lys	1.50 (2)	0.12
pmol anal	18	100			

[a] Residues per mole of peptide are given. The number of residues expected based on the sequence of angiogenin (Strydom et al., 1985) is given in parentheses. Quantities less than 0.1 residues are not indicated. Analyses are not corrected for Glx, Ser, and Gly which are present in some HPLC fractions at this level. Cystine and tryptophan contents were not determined.

Enzymatic Activity of ARH-II. The enzymatic activity of ARH-II was compared to those of angiogenin and RNase by using a variety of substrates. With tRNA at pH 6.8 (conditions optimal for angiogenin), ARH-II is 12-fold more active than angiogenin (Figure 4). With wheat germ RNA at pH 8.1 (conditions optimal for RNase), a 50-fold enhancement in activity was observed for ARH-II relative to angiogenin. With poly(C), and poly(U), 60- and 7-fold enhancements were observed, respectively. These activities are, however, still at

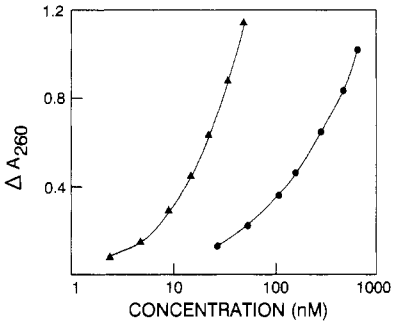

FIGURE 4: Cleavage of tRNA by ARH-II (▲) and angiogenin (●). Assays were carried out in 33 mM Hepes, pH 7.0, containing 33 mM NaCl, 0.1 mg/mL HSA, and 2 mg/mL tRNA at 37 °C for 2 h (total volume 300 μL), and the extent of cleavage was determined as described (Shapiro et al., 1987).

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

substrate	k_{cat}/K_m (M^{-1} s^{-1})			
	ARH-II[a]	ARH-IV[a]	angiogenin[a]	RNase A[b]
CpA	220	7180	12	9690000
UpA	75	453	1	3880000
CpG	50	119	4	555000
UpG	2	18	0.4	235000

[a] Incubations were performed at 37 °C in 33 mM Mes (pH 5.9) containing 33 mM NaCl, 0.1 mg/mL HSA, 0.1 mM substrate, and 0.3–5.0 μM enzyme. At various times, aliquots (15–20 μL) were applied to a Waters Radial-PAK C18 column for quantitation of substrate and products (Shapiro et al., 1986a,b). 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] Activities measured spectrophotometrically (Witzel & Bernard, 1962) at 37 °C in 33 mM Mes (pH 5.9) containing 33 mM NaCl, 0.1 mM substrate, and 2–35 nM enzyme.

Table IV: Chemical Modification of ARH-II and Angiogenin[a]

reagent	$t_{1/2}$ (min)[b]	
	ARH-II	angiogenin
$NaCNBH_3/H_2CO$[c]	8	22
p-hydroxyphenylglyoxal[d]	7	13
bromoacetate[e]	28	45

[a] Assays were performed essentially as described previously (Shapiro et al., 1987). [b] $t_{1/2}$ represents the time required for 50% inactivation. [c] Incubation performed at 37 °C in 20 mM Hepes, pH 6, with 10 mM $NaCNBH_3$ and 2 mM H_2CO. [d] Incubation performed at 37 °C in 25 mM Hepes, pH 8.0, with 10 mM reagent. [e] Incubation performed at 37 °C in 25 mM sodium acetate, pH 5.5, with 30 mM bromoacetate.

least 3 orders of magnitude lower than those for RNase A.

Table III shows k_{cat}/K_m values for cleavage of four dinucleoside 3′,5′-phosphates by ARH-II, angiogenin, and RNase A. In all cases, ARH-II is more active than angiogenin. The enhancement is greatest with UpA (75-fold) and least with UpG (5-fold).

pH Dependence. The pH dependence of ARH-II was examined with tRNA as substrate. Optimal activity is observed near pH 7.1, which is slightly higher than for angiogenin (pH ~6.8).

Chemical Modification of ARH-II. Chemical modification of ARH-II was carried out to determine whether the structural changes introduced lead to alterations in the reactivity of essential lysine, histidine, and arginine residues. In all cases, ARH-II is inactivated faster than angiogenin (Table IV). The largest difference (3-fold) is found with reductive methylation. Inactivation by *p*-hydroxyphenylglyoxal or bromoacetate is about 2-fold faster than with angiogenin.

Table V: Angiogenic Activity of ARH-II and Angiogenin[a]

sample	dose (ng)	% (*n*)[b]
angiogenin	10	50 (12)
	1	47 (68)
	0.1	28 (39)
ARH-II	10	21 (82)
	1	26 (77)
	0.1	22 (32)

[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 of angiogenin and ARH-II were performed simultaneously. Control samples containing only water produced 14% positive responses (total 58 eggs). [b] The total number of assays performed for each sample, *n*, is given in parentheses.

Table VI: Enzymatic Activities of Angiogenin/RNase Hybrid Proteins and RNase A Relative to Angiogenin[a]

substrate	ARH-I	ARH-II	ARH-IV	RNase A
tRNA	300	20	942	5.5×10^4
wheat germ RNA	600	50	ND	5.0×10^5
poly(C)	190	70	ND	$>5.0 \times 10^5$
poly(U)	170	7	ND	4.0×10^4
CpA	237	18	600	8.1×10^5
UpA	220	75	453	3.9×10^6
CpG	13	13	30	1.4×10^5
UpG	28	5	45	5.9×10^5

[a] Values for angiogenin are set at 1.0. Activities for ARH-I are from a previous study (Harper & Vallee, 1989). Activities toward dinucleoside 3′,5′-phosphates were measured as described in the legend to Table III. Other assays were performed as described previously (Shapiro et al., 1986a,b, 1987).

Biological Activity of ARH-II. As shown in Table V, the angiogenic activity of ARH-II in the chick chorioallantoic membrane assay is strikingly diminished compared to that of angiogenin. Thus, even at 10 ng/egg, the percentage of positive responses is less than half that observed with angiogenin at 1 ng/egg.

Purification and Characterization of ARH-IV. Replacement of residues 58–70 in angiogenin by the corresponding segment of RNase A gives a hybrid protein (ARH-I) characterized by markedly increased enzymatic activity (Harper & Vallee, 1989). To examine the combined effects of regional mutations contained in ARH-I and ARH-II, a hybrid gene was constructed in which segments encoding residues 38–41 and 58–70 of angiogenin were both replaced by sequences encoding the corresponding regions of RNase A. The hybrid protein product, ARH-IV, was expressed as described for ARH-II with a yield of 300 μg/L of culture. The amino acid compositions of ARH-IV (Table I) and its tryptic peptides (not shown) were as expected, indicating that the four disulfide bonds[3] had formed correctly and that no unintended changes had been introduced.

Enzymatic Activity of ARH-IV. The enzymatic activities of ARV-IV toward one polynucleotide and four dinucleotide substrates (Table III) are all higher than those observed for any angiogenin/RNase hybrid examined to date. With tRNA as substrate, ARH-IV is about 1000-, 50-, and 3-fold more active than angiogenin, ARH-II, and ARH-I, respectively (Table VI). The activities of ARH-IV toward the dinucleotides CpA, UpA, UpG, and CpG are 600-, 450-, 45-, and 30-fold higher, respectively, than those of angiogenin. These activities are 2-fold (CpG) to 33-fold (CpA) greater than those measured with ARH-II and 2–3-fold greater than

[3] The RNase A segment 59–73 contains a disulfide bond joining Cys-65 and Cys-72.

those observed with ARH-I (Table VI).

Discussion

Despite the extensive sequence identity between angiogenin and the pancreatic ribonucleases, their enzymatic activities differ dramatically. Angiogenin is 10^5–10^6-fold less active than RNase A toward a variety of polynucleotides and dinucleotides commonly employed as substrates for RNase A (Shapiro et al., 1986b, 1988). The specificities of the two enzymes toward these substrates also differ: e.g., with dinucleotides, the order of reactivity is CpA > CpG > UpA > UpG for angiogenin and CpA > UpA > CpG > UpG for RNase A (Shapiro et al., 1988; Harper & Vallee, 1988). Despite its low activity toward conventional RNase substrates, angiogenin is as effective as RNase A at abolishing the capacity of ribosomes to support cell-free protein synthesis (St. Clair et al., 1987). However, angiogenin destroys ribosomal function by cleaving specifically at a limited number of sites in 18S rRNA, while RNase A degrades both the 28S and 18S RNA extensively (St. Clair et al., 1988).

The structural basis for the marked functional differences between angiogenin and RNase A is not readily apparent from their primary sequences. Virtually all of the important active-site residues of RNase A are conserved in angiogenin, including Gln-11, His-12, Lys-41, Thr-45, Glu-111, Asp-121, and Ser-123. Other structural components, however, may play crucial roles in conferring specificity and reactivity by maintaining the critical alignment of these active-site residues.

In the present study, the role of nonconserved residues flanking the conserved active-site residue Lys-40 has been examined. The essentiality of this lysine for both the enzymatic and angiogenic activities of angiogenin was established previously by site-directed mutagenesis (Shapiro et al., 1989). Thus, the K40Q angiogenin mutant has no detectable ribonucleolytic or angiogenic activity, and the K40R mutant displays greatly reduced enzymatic activity (2.2%) compared with angiogenin. These data are consistent with the inference from X-ray crystallographic studies that the ϵ-amino group of the corresponding lysine of RNase A serves to stabilize a pentacoordinate intermediate or transition state formed during the transesterification and hydrolysis reactions (Roberts et al., 1969; Richards & Wyckoff, 1971; Holmes et al., 1978; Alber et al., 1983). In the computed three-dimensional structure of angiogenin (Palmer et al., 1986), Lys-40 occupies a position at least grossly comparable to that of its counterpart in the pancreatic RNase. However, this model reveals substantial differences in the secondary structures of the two proteins in this region which may influence the precise orientation of the lysine and/or other nearby critical residues. For example, Lys-40 is preceded by a four-residue type III β-turn located on the surface of the protein, while the corresponding segment of RNase A forms a type I β-turn.

The primary structures of angiogenin and pancreatic RNase in this region differ in major respects (Figure 1). The proline residue immediately following Lys-41 in RNase A is replaced by aspartic acid in angiogenin. This proline is conserved in 39 of the 41 pancreatic RNases whose sequences have been reported; the remainder have alanine or phenylalanine (Beintema et al., 1986). In addition, the Asp-Arg sequence at positions 38–39 in RNase A is replaced by a single amino acid, proline, in angiogenin. Although the residues at these two positions vary considerably among the pancreatic RNases (Beintema et al., 1986), in no instance is there a deletion or a proline. These replacements underlie the differences in β-turn structure described above. The conservation of Pro-38, which is primarily responsible for the type III β-turn in angiogenin, in all five mammalian angiogenins sequenced to date (Bond & Strydom, 1989; M. D. Bond and D. J. Strydom, personal communication) suggests that this particular β-turn may be functionally critical.

These considerations served as the basis for designing the hybrid protein, ARH-II, in which residues 38–41 of angiogenin have been replaced by the corresponding RNase A segment (residues 38–42) (Figure 1). ARH-II displays markedly enhanced activity toward both polynucleotide and dinucleoside 3′,5′-phosphate substrates, with the largest increases (50–75-fold) found with poly(C), UpA, and wheat germ RNA (Table VI). Thus, the precise sequence in the region around the active-site lysine appears to be important in attenuating angiogenin's overall reactivity toward conventional pancreatic RNase substrates. The increased activity of ARH-II compared with angiogenin may result from a more optimal positioning of the ϵ-amino group of the lysine itself, induced by the flanking residues. However, it may also reflect influences of the regional replacement on the spatial orientations of other active-site components, located outside the modified segment. An attractive candidate in this regard is Thr-44. Crystallographic analyses and molecular modeling studies of RNase–substrate analogue complexes suggest that the corresponding residue in RNase A (Thr-45) makes two important H bonds with the pyrimidine ring in CpN and UpN substrates: backbone NH to O(2) and side-chain OH to N(3) (Brünger et al., 1985; Richards & Wyckoff, 1971; Wodak et al., 1977). It is possible that changes in adjacent residues around Lys-40 in angiogenin may attenuate the effectiveness of Thr-44 in fulfilling this substrate binding role. The regional replacement in ARH-II may then reposition the threonine side chain and/or backbone so as to facilitate the formation of stronger hydrogen-bonding interactions with the substrate. The differences in discrimination between CpN and UpN substrates by angiogenin as compared with ARH-II (and RNase A) (Table III) suggest that the active-site threonine indeed functions differently in the two enzymes.

Clearly, the gross differences in the enzymatic activities of angiogenin and RNase are not associated with a single amino acid or even a single region. The activity of ARH–II, although significantly more RNase A–like than that of angiogenin, remains at least 3 orders of magnitude lower than that of RNase (Tables III and VI). ARH–I (Harper & Vallee, 1989), which contains the putative purine binding region of RNase A, also displays markedly enhanced pancreatic RNase–like activity but is still 10^2–10^4-fold less potent than RNase A. A double regional mutant, ARH-IV, containing the RNase segments of both ARH-I and ARH-II, was created in order to determine whether the combined replacements would yield an enzyme with a specific activity toward some conventional RNase substrate approaching that of RNase A, as might be expected if the effects of the two individual replacements are independent. ARH-IV activity, however, is only 2–3-fold greater than that of ARH-I (Table VI). This may reflect the presence in the double mutant of a new, detrimental interaction that reduces the catalytic potential inherent in each individual mutant. Alternatively, some of the structural effects of the two regional replacements responsible for the increased catalytic efficiency may be similar or identical. For example, although the two modified segments are relatively far apart in the calculated angiogenin structure (Palmer et al., 1986), they both flank a strand of β-sheet structure that contains Thr-44, and it is possible that both regional mutations increase the capacity of this residue to form hydrogen bonds with the substrate (see above). Since the free energy advantage of such

interactions can be contributed only once in the double regional mutant, the activity observed would be much less than anticipated if the two regional replacements enhance catalysis by independent mechanisms.

Several studies have now explored the relationship between angiogenin's enzymatic and angiogenic activities. Both activities are abolished by placental ribonuclease inhibitor (Shapiro & Vallee, 1987), which binds extremely tightly to angiogenin ($K_i = 7 \times 10^{-16}$ M) (Lee et al., 1989). Mutation of any one of the active-site residues His-13, His-114, or Lys-40 also abolishes both activities, implying that an intact active site is necessary for angiogenesis (Shapiro et al., 1989; Shapiro & Vallee, 1989). In addition, changing Asp-116 to histidine enhances ribonucleolytic activity by 18-fold and angiogenic potency by 1–2 orders of magnitude (Harper & Vallee, 1988), again indicating that the two activities are interrelated.

With both ARH-I (Harper & Vallee, 1989) and now ARH-II, however, there is an apparent dissociation of enzymatic and biological activities: the hybrids have markedly increased ribonucleolytic activity, but greatly reduced angiogenic potency, relative to angiogenin. This loss of angiogenic activity could reflect alterations in enzymatic specificity, which make the hybrids more akin to pancreatic RNase (Tables III and VI). Thus, the biological action of angiogenin may require a specific cleavage of some RNA or RNA-like substrate which the hybrids are less able to perform. The D116H mutant noted above, which has increased enzymatic *and* angiogenic activity, appears to retain more of the specificity of angiogenin (Harper & Vallee, 1988).

The diminished angiogenic activity of ARH-I and ARH-II might also be accounted for by their inability to bind effectively to cellular targets. There is now considerable evidence that the action of angiogenin is mediated through a receptor. This evidence includes (i) the multiplicity of responses elicited by angiogenin when added to cultured cells (Bicknell & Vallee, 1988, 1989; Heath et al., 1989; Moore & Riordan, 1990), (ii) the capacity of the inactive H13A and H114A mutants to inhibit the angiogenic activity of angiogenin (Shapiro & Vallee, 1989), and (iii) the high-affinity, specific binding of angiogenin to calf pulmonary artery endothelial cells (Badet et al., 1989). In ARH-I and ARH-II, some angiogenin residues that participate directly in receptor binding may have been lost or repositioned. Alternatively, the replacements introduced in the hybrids may produce conformational perturbations in receptor binding regions located outside these segments.

Finally, it should be noted that neither the physiological substrate(s) of angiogenin nor the putative angiogenin receptor have yet been identified. It is possible that the receptor itself contains an RNA-related moiety that is cleaved by, or merely binds to, angiogenin. In this case, the two explanations for the decreased angiogenicity of ARH-I and ARH-II delineated above—changes in enzymatic specificity and attenuated receptor binding—may be essentially equivalent.

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