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Replacement of Residues 8-22 of Angiogenin with 7-21 of RNase A Selectively Affects Protein Synthesis Inhibition and Angiogenesis†

Michael D. Bond and Bert L. Vallee*
Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, Boston, Massachusetts 02115
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ABSTRACT: The region of human angiogenin containing residues 8-21 is highly conserved in angiogenins from four mammalian species but differs substantially from the corresponding region of the homologous protein ribonuclease A (RNase A). Regional mutagenesis has been employed to replace this segment of angiogenin with the corresponding RNase A sequence, and the activities of the resulting covalent angiogenin/RNase hybrid, designated ARH-III, have been examined. The ribonucleolytic activity of ARH-III is unchanged toward most substrates, including tRNA, naked 18S and 28S rRNA, CpA, CpG, UpA, and UpG. In contrast, the capacity of ARH-III to inhibit cell-free protein synthesis is decreased 20-30-fold compared to that of angiogenin. The angiogenic activity of ARH-III is also different; it is actually more potent. It induces a maximal response in the chick chorioallantoic membrane assay at 0.1 ng per egg, a 10-fold lower dose than required for angiogenin. In addition, binding of ARH-III to the placental ribonuclease inhibitor is increased by at least 1 order of magnitude ($K_i \leq 7 \times 10^{-17}$ M) compared to angiogenin. Thus, mutation of a highly conserved region of angiogenin markedly affects those properties likely involved in its biological function(s); it does not, however, alter ribonucleolytic activity toward most substrates.

Angiogenin is a potent inducer of blood vessel growth in the chick chorioallantoic membrane (CAM)[1] and rabbit cornea assays (Fett et al., 1985). In vitro, the protein induces multiple responses in endothelial cells including activation of phospholipase C and secretion of prostacyclins (Bicknell & Vallee, 1988, 1989); it also inhibits cell-free protein translation by specific cleavage of 18S RNA within the 40S ribosomal subunit (St. Clair et al., 1987, 1988). Angiogenin is homologous to the ribonuclease family of enzymes. It has 33% sequence identity to the most extensively studied member of this group, bovine pancreatic RNase A (Strydom et al., 1985; Kurachi et al., 1985); its tertiary structure is similar as well, based on conservation of three of four disulfide bonds, extremely tight binding to the common ligand PRI (Shapiro & Vallee, 1987; Lee et al., 1988b; Blackburn et al., 1977), and a computer-generated three-dimensional structure (Palmer et al., 1986). The three essential catalytic residues of RNase A are conserved in angiogenin (His-13, Lys-40, and His-114) as are many other key active-site and structural residues. Indeed, angiogenin possesses ribonucleolytic activity, but of a type far different from other RNases; its activities toward most substrates are 5-6 orders of magnitude below those of RNase A (Shapiro et al., 1987, 1988; Harper & Vallee, 1989).

The vast difference in in vivo and in vitro activities of angiogenin compared to the RNases (which have not been shown to induce angiogenesis or second-messenger activities) is clearly indicative of a distinct physiological role for this protein. Two lines of evidence implicate the amino-terminal region of angiogenin in this function. The first comes from experiments with noncovalent hybrids formed between synthetic angiogenin peptides and fragments of RNase A (Harper et al., 1988). Maximal activities of both the Ang(1-21)/RNase(21-124)[2] and the RNase(1-118)/Ang(108-123) hybrids toward small substrates are decreased compared to those of RNase A. Of the two hybrids, however, only the first is able to generate the pattern of cleavage products from 18S and 28S rRNA that is characteristic of angiogenin. Therefore, the amino-terminal region of angiogenin may be involved in determining specificity.

The second line of evidence comes from a comparison of the sequences of angiogenins from four different species. There

† This work was supported by funds from Hoechst, A.G., under an agreement with Harvard University.
* Address correspondence to this author at the Center for Biochemical and Biophysical Sciences and Medicine, Seeley G. Mudd Building, 250 Longwood Ave., Boston, MA 02115.

[1] Abbreviations: ARH-III, angiogenin/RNase hybrid in which residues 8-22 of angiogenin are replaced by 7-21 of RNase A; RNase, ribonuclease; RNase A, bovine pancreatic ribonuclease A; PRI, placental ribonuclease inhibitor; C18, octadecylsilane; CAM, chorioallantoic membrane; TFA, trifluoroacetic acid; CpA, cytidylyl (3′-5′)adenosine; CpG, cytidylyl(3′-5′)guanosine; UpA, uridylyl(3′-5′)adenosine; UpG, uridylyl(3′-5′)guanosine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol.

[2] Ang(n-n') refers to an angiogenin peptide whose N- and C-terminal residues are denoted by n and n', respectively, indicating their positions in the primary structure of angiogenin. The same terminology is used for RNase A fragments.

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is a striking degree of identity within the first 21 amino acids of these proteins (Bond & Vallee, 1988; Bond and Vallee, unpublished results; Maes et al., 1988), particularly in the block of residues from His-8 to Arg-21 (human angiogenin numbering). Of 14 residues in this segment, 13 are completely conserved in the human, bovine, lapine, and porcine proteins. Importantly, the segment differs significantly from the corresponding sequence in RNase A. Thus, these residues might provide a biological capacity or specificity for angiogenin beyond mere degradation of RNA.

Studies were therefore undertaken to investigate the functional role of the N-terminal segment of angiogenin using regional mutagenesis. Recombinant DNA methodologies were employed to design and express a covalent hybrid protein, ARH-III,[3] in which residues 8–22 of angiogenin are replaced by the corresponding sequence from RNase A. The effects of this substitution on the enzymatic and biological properties of the protein have been examined.

Experimental Procedures

Materials. Materials were obtained from the sources listed in Harper and Vallee (1989) and Bond and Vallee (1988). The angiogenin expression plasmid pAng2, provided by R. Shapiro, contains a modified Trp promoter and ampicillinase marker for selection (Shapiro et al., 1988). DNA sequencing was performed with a Sequenase kit (U.S. Biochemical Corp.) with deoxyadenosine 5′-(α-[^{35}S]thiotriphosphate). Oligodeoxynucleotides were obtained from the Biopolymer Laboratory, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School. Unless otherwise noted, angiogenin was obtained from genetically engineered baby hamster kidney cells (Kurachi et al., 1988); quantitation of angiogenin and ARH-III was by amino acid analysis.

Construction of the ARH-III Coding Sequence. The coding sequence for ARH-III (Figure 2a,b) was constructed as shown in Figure 3). Fragments A and C were generated by cleavage of pAng2 DNA (13 μg) with either *Xho*I and *Eco*RI, or *Pvu*I and *Eco*RI, followed by purification by electrophoresis in 3% gels using NuSieve GTG low melting point agarose. Fragment A (2928 bp) contained the entire noncoding region of the plasmid and the coding region for Met-(−1) through Asn-3; fragment C (306 bp) consisted of the coding region from Arg-24 through Pro-123 and the two stop codons. Fragments A and C were then ligated with fragment B, a synthetic double-stranded oligodeoxynucleotide coding for residues 4–7 of angiogenin, residues 7–21 of RNase A, and residue 23 of angiogenin (Figure 2b). The duplex was obtained by annealing the 5′-phosphorylated complementary oligomers as described (Harper & Vallee, 1989). The most successful ligation reaction employed 24, 150, and 100 pmol of fragments A, B, and C, respectively, and 6 units of T4 DNA ligase in a 60-μL volume; the final agarose concentration was 1.4%. *Escherichia coli* strain W3110 cells were then transformed by using the $CaCl_2$ procedure (Maniatis et al., 1982), and plasmid uptake was detected by ampicillin resistance.

Both restriction enzyme mapping (with *Hae*III or with *Eco*RI and *Kpn*I) and nucleotide sequencing were employed to ensure that the new plasmid, designated pARH-III (ATCC designation 68188), contained the correct coding sequence. For sequencing, the *Kpn*I/*Eco*RI fragment containing the coding region was isolated and cloned into M13mp18 by using standard procedures. The nucleotide sequence corresponding to the first 60 amino acids of the protein was as expected.

[3] ARH-I has been described previously (Harper & Vallee, 1989).

Expression and Purification of ARH-III. After selection of the most productive clone, ARH-III was expressed, refolded, and then purified by Mono S cation-exchange and C18 HPLC, as described (Shapiro et al., 1988).

Removal of Met-(−1). The Met-(−1) residue of the hybrid protein (9 μM) was removed enzymatically as described by Shapiro et al. (1988) under conditions favorable for the spontaneous cyclization of the next residue, Gln-1, to pyroglutamic acid. The protein was then purified by reversed-phase HPLC as above, and peak fractions were pooled and dialyzed against water.

Structural Characterization of ARH-III. Amino acid analysis was performed as described (Strydom et al., 1985) using a Picotag derivatization method and Waters Associates HPLC system. Trypsin digestion was accomplished by incubating 4.1 nmol of ARH-III with 95 pmol of HPLC-purified trypsin in 730 μL of 20 mM Tris/0.35 M NaCl, pH 8.0, for 24 h at 37 °C. The peptides were separated by reversed-phase HPLC as described (Shapiro et al., 1988) and identified by elution position and amino acid composition.

SDS–PAGE was performed on 15% polyacrylamide gels with 5% stacking gels as described by Laemmli (1970). Proteins were visualized by using an ICN rapid silver staining kit.

Enzymatic Assays. Activity toward yeast tRNA was determined by using a precipitation assay (Shapiro et al., 1987) with angiogenin or ARH-III concentrations of 18–430 nM. Assays for the pH profiles were performed by using the following buffers with pH values at 37 °C: Mes, pH 5.4, 5.8, and 6.3; Hepes, pH 6.2, 6.8, 7.3, and 7.8; Tris, pH 7.5, 8.1, and 8.5; Ches, pH 8.8, 9.3, and 9.9. The pH values were calculated from the readings at 25 °C using $\Delta pK_a/°C$ values supplied by Research Organics.

Activity toward dinucleoside 3′,5′-phosphates was determined by an HPLC quantitation method (Shapiro et al., 1988; Harper & Vallee, 1989). Reaction mixtures (30 μL) contained 0.1 mM substrate and 2.8 μM ARH-III and were incubated for 4–24 h at 37 °C.

Ribonucleolytic activity toward 18S and 28S rRNA was visualized as described (Shapiro et al., 1986) with calf liver rRNA (Pharmacia) as substrate.

Inhibition of in Vitro Protein Synthesis and Cleavage of Intact Ribosomes. The effect of ARH-III on protein translation in rabbit reticulocyte lysate was determined in a modification of the procedure of St. Clair et al. (1987) as described by Harper and Vallee (1989). Reaction mixtures contained 10–210 nM angiogenin or ARH-III.

ARH-III-catalyzed cleavage of reticulocyte ribosomal RNA in intact ribosomes was examined by using the method of St. Clair et al. (1987) as described (Harper & Vallee, 1989).

Biological Activity. Angiogenesis was assessed by using the chick embryo CAM assay of Knighton et al. (1977), as described (Fett et al., 1985).

Binding of ARH-III to PRI. The apparent second-order association constant, k_a, was determined by examining the competition between ARH-III and RNase A for PRI, as described (Lee & Vallee, 1989a; Lee et al., 1989a). The interaction was monitored in 0.1 M Mes, 0.1 M NaCl, and 1 mM EDTA, pH 6.0 at 25 °C. The k_a value obtained for ARH-III is the average of seven separate determinations.

The dissociation rate constant, k_d, was determined by monitoring the release of ARH-III from the protein–inhibitor complex as a function of time in the presence of excess scavenger for free PRI (Lee & Vallee, 1989a; Lee et al., 1989b). Free ARH-III was measured by cation-exchange HPLC over

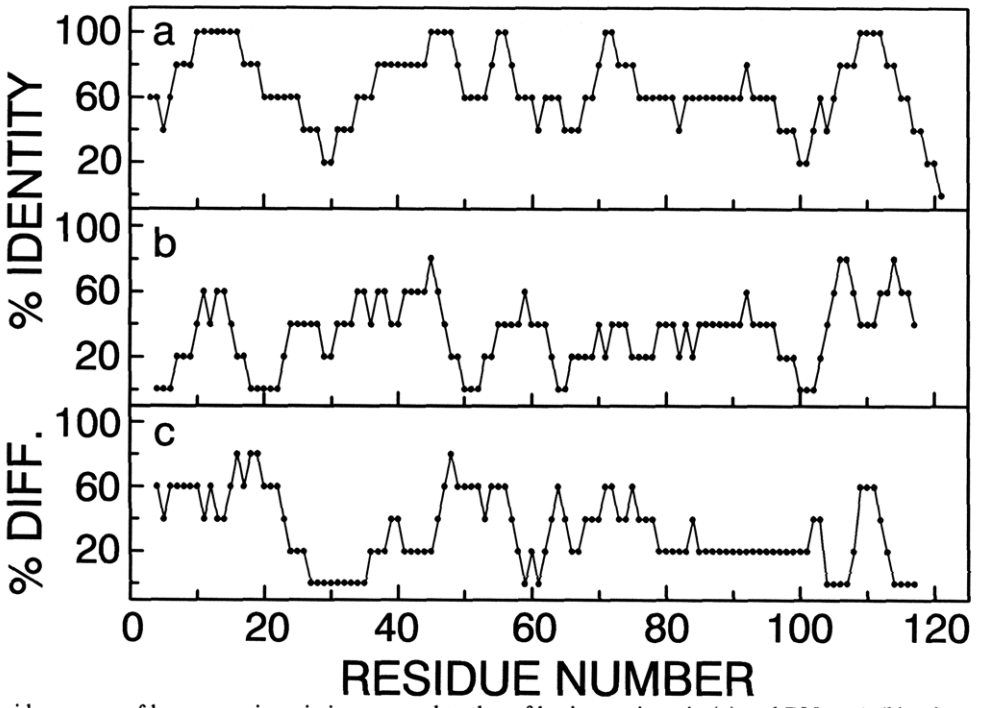

FIGURE 1: Amino acid sequence of human angiogenin is compared to that of bovine angiogenin (a) and RNase A (b) using a computer program written by D. J. Strydom. The plots show the percent identity between two sequences within a five-residue window, centered at the residue of human angiogenin indicated. In order to facilitate alignment of the sequences, the N-terminal Ala of bovine angiogenin was deleted, as were RNase A residues Asn-24, Arg-39, Gln-69, Thr-70, Pro-114, and Tyr-115 (RNase A numbering). (c) Difference in percent identity between plots a and b; values from plot b were subtracted from corresponding values in plot a.

a 10-day period. The incubation was performed at 25 °C in the above buffer containing 120 μM DTT.

Results

Similarity Plots. Similarity plots were computed to provide a quantitative means of comparing the sequence of human angiogenin with that of bovine angiogenin (Figure 1a) and RNase A (Figure 1b). The plots show the percent identity between two proteins within a moving five-residue window centered at the human angiogenin residue indicated. Because values are averaged over five residues, the plots emphasize regional trends rather than similarities/differences at individual sites.

The first plot (Figure 1a) compares the sequence of human angiogenin with that of bovine angiogenin (Maes et al., 1988; Bond & Strydom, 1989). In most instances, the identity is 60% or greater, consistent with an overall identity of 64%. Not surprisingly, the regions around the three essential catalytic residues of angiogenin, His-13, Lys-40, and His-114, are highly similar between the proteins; in fact, the three longest segments of 80% identity or higher occur at positions 7–19, 37–48, and 106–114. Five different regions show 100% identity, and the longest is that segment from positions 10–16.

Figure 1b compares human angiogenin with RNase A. The degree of similarity is much lower in general than that observed above, consistent with the overall sequence identity of 33%. In this case, it is actually the differences between the two sequences that holds the most interest, since they may underlie the different biological roles of these proteins. Short regions of least similarity (0% identity) occur at positions 4–6, 50–52, 64–65, and 100–102; the longest region is from positions 18–22.

Figure 1c shows the difference of the two above figures, calculated by subtracting plot 1b from 1a. High values indicate areas of human angiogenin with *both* high identity to bovine angiogenin and low identity to RNase A. These are regions most likely to be involved in a unique role for angiogenin. Only four individual windows reach a difference in identity of 80%, and three of them occur in close proximity at positions 16, 18, and 19. Thus, this one small segment stands out as the most conserved in the angiogenins and the most different from RNase A. The individual residues encompassed in this segment, including all those within the two end windows, are Tyr-14 through Arg-21.

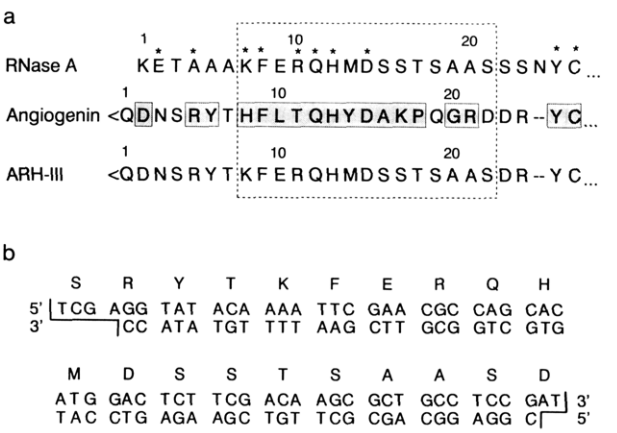

FIGURE 2: (a) Amino-terminal sequences of RNase A, human angiogenin, and ARH-III. Residues in RNase A conserved in pancreatic RNases from 39 mammalian species (Beintema et al., 1986) are indicated by an asterisk; boxed residues in angiogenin are conserved in the human, bovine (Bond & Vallee, 1988; Maes et al., 1988), porcine, and lapine proteins (unpublished results). <Q is pyroglutamic acid. (b) Synthetic oligonucleotide duplex (fragment B) encoding residues 4–23 of ARH-III; the termini are compatible for ligation of *Xho*I and *Pvu*I restriction enzyme cleavage sites.

Construction of the ARH-III Coding Sequence. The entire section of angiogenin selected for mutagenesis extends from His-8 to Asp-22 (see Discussion); these residues are replaced by residues 7–21 of RNase A (Figure 2). The DNA coding sequence for the hybrid protein, designated ARH-III, was constructed as shown in Figure 3. The presence of the desired coding sequence in the genetically engineered plasmids was

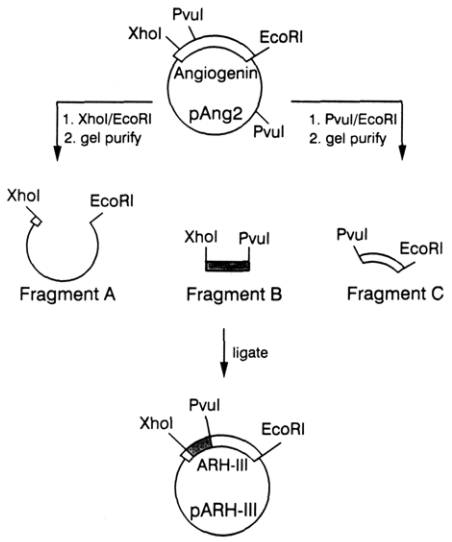

FIGURE 3: Construction of the coding sequence for ARH-III beginning with the pAng2 expression plasmid for angiogenin; details are given under Experimental Procedures. Fragment B is the synthetic duplex from Figure 2b.

confirmed by restriction enzyme mapping and by nucleotide sequence analysis.

Expression and Purification of Met-(−1) ARH-III. The hybrid protein was expressed in *E. coli* and purified by using Mono S cation-exchange and C18 reversed-phase HPLC. The hybrid eluted earlier than angiogenin on the Mono S column (8-min difference in retention time, 50-min gradient from 0.15 to 0.55 M NaCl in 10 mM Tris, pH 8.0). It eluted as a sharp, symmetrical peak on the C18 column with a small shoulder comprising 5% of the total area. The central fractions of the major peak were pooled and dialyzed, and SDS–PAGE analysis revealed a single compact band migrating at $M_r \sim$ 14 500. The final yield was 0.4 mg/L culture.

Removal of Met-(−1). Angiogenin expressed in *E. coli* using pAng2 contains Met-(−1) at the amino terminus. To date, the extra residue has not been found to affect enzymatic or angiogenic activity. Nevertheless, because of its proximity to the replaced region, the amino-terminal Met-(−1) residue of ARH-III was removed by digestion with *Aeromonas* aminopeptidase. Amino acid composition and peptide mapping indicate that removal of Met-(−1) was greater than 95% (see below).

Structural Characterization of ARH-III. The amino acid composition of ARH-III is consistent with the successful replacement of residues 8–22, as well as the removal of Met-(−1) (Table I).

Results of a tryptic digest of ARH-III also support the successful replacement (Table I and Figure 4). Individual peptides identified by amino acid composition accounted for the entire sequence. All three cystine-linked peptide pairs, T-9b, T-10, and T-11 [nomenclature of Strydom et al. (1985)], were recovered, consistent with the proper refolding and oxidation of the protein. Peptides T-1, -2, -3a, -3b, -4a, -5, -6, and -8 and disulfide-linked T-10 and T-11 had compositions and elution positions essentially the same as those observed for human angiogenin (Strydom et al., 1985; Shapiro et al., 1988). The amino-terminal peptide T-1 was recovered in >95% yield, again indicating essentially complete removal of the Met-(−1).

As expected, the only differences between ARH-III and angiogenin occur with peptides T-7 and T-9′ (Figure 4). In their places, the hybrid gives rise to two new tripeptides, designated T-7a and T-7b, and an extended form of T-9′,

Table I: Amino Acid Composition of ARH-III and Its Tryptic Peptides[a]

amino acid	angiogenin	ARH-III	T-7b	T-7ab	T-9b
Asp	15	14.0 (14)	0.14		2.19 (2)
Glu	10	9.9 (10)	1.00 (1)	0.98 (1)	2.95 (3)
Ser	9	12.6 (13)	0.12		6.56 (7)
Gly	8	7.5 (7)	0.12		0.52
His	6	4.9 (5)			1.11 (1)
Arg	13	13.3 (13)	1.00 (1)	1.03 (1)	2.17 (2)
Thr	7	7.3 (7)		1.00 (1)	2.87 (3)
Ala	5	6.1 (6)			1.87 (2)
Pro	8	7.5 (7)			0.18
Tyr	4	2.7 (3)		0.95 (1)	0.86 (1)
Val	5	4.0 (5)			0.94 (1)
Met	1	2.0 (2)			1.81 (2)
Ile	7	6.5 (7)			1.03 (1)
Leu	6	5.3 (5)		0.1	0.15
Phe	5	4.9 (5)	0.90 (1)	0.90 (1)	0.98 (1)
Lys	7	7.1 (7)		1.04 (1)	1.23 (1)
Cys	6	6 (6)			2 (2)
pmol			520	450	170

[a] Values are given as residues per mole; quantities less than 0.1 residue per mole are not shown. The number of residues predicted for the ARH-III sequence are shown in parentheses. Tryptic peptide compositions are from 200-μL aliquots of individual column fractions; picomoles of peptide per 200 μL is shown at the bottom. ARH-III Cys results are inferred from coelution of disulfide-linked peptides after tryptic digestion.

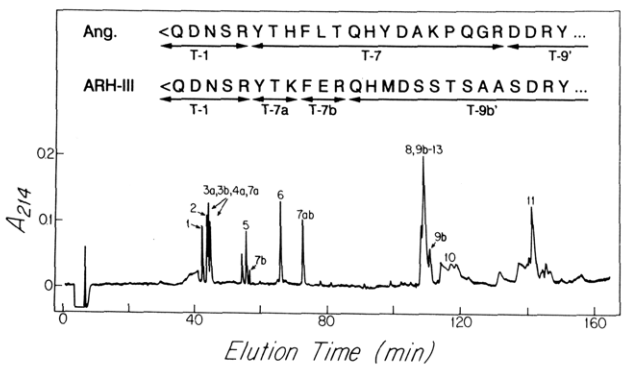

FIGURE 4: Separation of tryptic peptides of ARH-III. An Altex Ultrasphere IP C18 column was used with a linear gradient from 0 to 50% solvent B over 140 min (solvent A = 0.1% TFA; solvent B = 3:2:2 2-propanol/acetonitrile/0.08% aqueous TFA); the flow rate was 0.8 mL/min. Peptide designations for angiogenin are from Strydom et al. (1985). The amino-terminal sequences of angiogenin and ARH-III are shown; double-headed arrows indicate peptides generated by tryptic cleavage.

designated T-9b′. T-7a eluted with three other peptides in two or more poorly resolved peaks at 44 and 45 min; they were identified by amino acid composition (not shown). Peptide T-7b eluted as a discrete peak at 56 min (Figure 4, Table I). The unhydrolyzed hexapeptide constituting T-7ab was also recovered (Figure 4; Table I). T-9b′, which is disulfide-linked to T-9″ to form the double peptide designated T-9b, was detected in two forms. First, the linked peptide T-9b eluted in essentially pure form in the peak at 111 min (Figure 4, Table I). Second, the large peak at 109 min contains a mixture of T-8, T-9b, and one to two Arg residues. This is consistent with the incomplete cleavage of T-13 (an Arg-Arg dipeptide) from the carboxyl terminus of T-9b′, and coelution of the T-9b′-13/T-9″ double peptide with T-8.

Enzymatic Activity. The enzymatic activities of ARH-III toward tRNA, 18S and 28S rRNA, and dinucleotide substrates are very similar to those of angiogenin. In the tRNA assay, both proteins exhibit a nonlinear response for ΔA_{260} versus enzyme concentration, and their activities are indis-

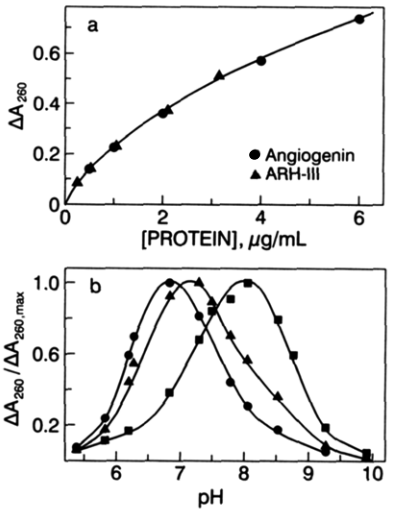

FIGURE 5: (a) Activity of angiogenin and ARH-III toward yeast tRNA. The assay measures the generation of acid-soluble fragments of tRNA at different concentrations of ARH-III and angiogenin (2-h incubation, pH 6.8, 37 °C). (b) Effects of pH on activities of ARH-III (▲), angiogenin (●), and RNase A (■) toward yeast tRNA. For comparison, activities are normalized to the maximum ΔA_{260} obtained for each protein.

Table II: Activity of ARH-III toward Dinucleotide Substrates[a]

substrate	k_{cat}/K_m (M^{-1} s^{-1})		
	ARH-III	angiogenin[b]	RNase A[c]
CpA	13	12	6.0×10^6
CpG	3.4	4.0	5.1×10^5
UpA	0.8	1.1	4.0×10^6
UpG	0.4	0.4	1.8×10^5

[a] Assays with ARH-III and angiogenin were performed in 30 mM Mes/30 mM NaCl, pH 6.0 at 37 °C. [b] Values from Shapiro et al. (1988) and Harper and Vallee (1989). [c] Values from Harper and Vallee (1989); assays performed spectrophotometrically in above buffer at 25 °C.

tinguishable at the concentrations tested (Figure 5a). ARH-III does differ somewhat from angiogenin, however, in that its pH optimum is ~7.3 (Figure 5b). Angiogenin and RNase A have maximal activities under these assay conditions at pH ~6.8 and ~8.1, respectively, consistent with previous results (Lee & Vallee, 1989b).

Angiogenin catalyzes the cleavage of dinucleotide substrates, but at rates 5–6 orders of magnitude lower than those observed for RNase A (Shapiro et al., 1988; Harper & Vallee, 1989). Its specificity is different from that of RNase A based on k_{cat}/K_m values for four different dinucleotides. The activity of ARH-III toward these substrates is very similar to that of angiogenin (Table II). In all cases, k_{cat}/K_m values are within 30% of those observed for angiogenin, and the relative specificity for the substrates is unchanged.

Angiogenin cleaves naked 18S and 28S rRNA in a selective manner, yielding products of approximately 100–500 nucleotides in length which remain resistant to further degradation for some time (Shapiro et al., 1986). The activity of ARH-III toward this substrate is indistinguishable from that of angiogenin (data not shown). In time points taken over 60–90 min, the two proteins (0.67 μM) catalyzed the formation of products of similar size and intensity, as revealed by agarose gel electrophoresis.

Effects on in Vitro Protein Synthesis. Angiogenin inhibits translation in rabbit reticulocyte lysate by catalyzing a highly specific cleavage of 18S rRNA in the 40S ribosoomal subunit, generating a small number of discrete products (St. Clair et al., 1987, 1988). RNase A, on the other hand, attacks the ribosome at numerous sites to form a huge number of reaction products of varying sizes. Of the two, angiogenin is actually more effective at inactivating the ribosomes, even though its rate with other RNA substrates is up to a million times slower than that of RNase A. This remarkable specificity is observed only with intact ribosomes or ribosomal subunits, and not with isolated 18S and 28S rRNA.

Table III: Inhibition of in Vitro Protein Translation by ARH-III, Angiogenin, and RNase A[a]

sample	concentration (nM)	% inhibition
ARH-III	70	0
	210	16
	210[b]	44
angiogenin	10	15
	30	86
	70	100
RNase A	30	30
	70	99

[a] Assays performed in rabbit reticulocyte lysate system as described under Experimental Procedures. Percent inhibition based on amount of ^{35}S-labeled protein recovered by acid precipitation; 0% and 100% inhibition correspond to 61 000 and 200 cpm, respectively. [b] Reaction time of ARH-III with lysate mixture was 45 min instead of the usual 15 min.

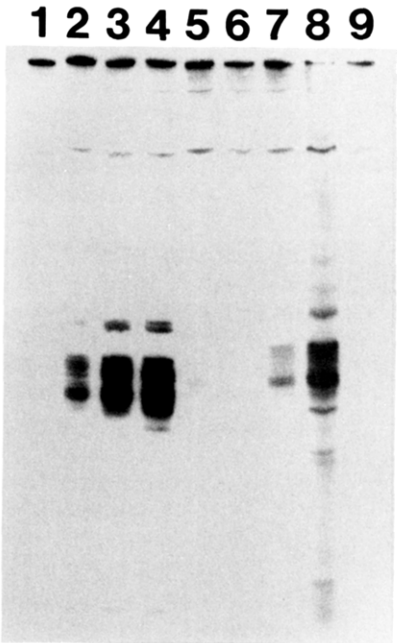

FIGURE 6: Effects of angiogenin and ARH-III on intact ribosomes. Rabbit reticulocyte lysate was incubated for 15 min with H_2O (lanes 1 and 9); angiogenin at 10 nM (lane 2), 30 nM (lane 3), and 70 nM (lane 4); or ARH-III at 30 nM (lane 5), 70 nM (lane 6), 210 nM (lane 7), and 210 nM with a 45-min incubation (lane 8). The rRNA was then purified by phenol/chloroform extraction and ethanol precipitation, 5′ labeled with [γ-^{32}P]ATP, electrophoresed on a 10% polyacrylamide/urea gel, and visualized by autoradiography. Samples in lanes 1–4 were loaded and electrophoresed for 5 min before loading lanes 5–9.

ARH-III is markedly less effective than angiogenin in inhibiting protein synthesis in rabbit reticulocyte lysate (Table III). Whereas angiogenin inhibits protein translation completely at a concentration of 40–70 nM, ARH-III at 210 nM produces less than 50% inhibition, even with a 3-fold longer incubation time. A comparison of the amounts of protein required to generate similar levels of inhibition indicates that ARH-III is 20–30-fold less active than angiogenin in this assay system.

In order to determine whether ARH-III cleaves ribosomal RNA in the same selective manner as angiogenin, rRNA from

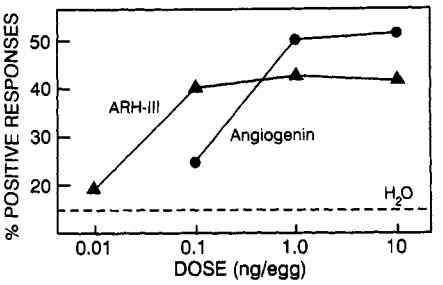

FIGURE 7: Angiogenic activity of ARH-III and angiogenin in the CAM assay. Individual assays employed sets of 10–20 eggs. The total number of eggs used was 248 for ARH-III, 125 for angiogenin, and 20 for H_2O. Angiogenin was from an *E. coli* expression system and contained a Met-(−1) residue; its potency is indistinguishable from that of native angiogenin containing pyroglutamic acid at the amino terminus (Shapiro et al., 1988).

Table IV: Binding Constants for ARH-III with PRI[a]

sample	k_a (M^{-1} s^{-1} × 10^{-8})	k_d (s^{-1} × 10^7)	K_i (fM)
ARH-III	4.3	≤0.3	≤0.07
angiogenin[b]	1.8	1.3	0.7
RNase A[b]	3.4	150	40

[a] Incubation conditions were 0.1 M Mes, 0.1 M NaCl, and 1 mM EDTA, pH 6.0, 25 °C. [b] From Lee et al. (1989b).

the above reaction mixtures was isolated, radiolabeled, and analyzed by urea/polyacrylamide gel electrophoresis (Figure 6). ARH-III generates essentially the same pattern of cleavage products as angiogenin, but requires much higher concentrations to do so. Thus, it takes a 45-min incubation time for 210 nM ARH-III to yield products with the same intensity as those generated by 20 nM angiogenin with a 15-min incubation time. This is consistent with a 30-fold difference in activity.

Angiogenic Activity. The dose response of ARH-III in the CAM assays is different from that of angiogenin (Figure 7). Angiogenin typically approaches maximal activity at a dose of approximately 1 ng, inducing a positive response in 50–60% of the eggs. Its activity at 0.1 ng is usually close to the 15–20% positive background level observed with water alone (J. W. Fett, personal communication). In contrast, ARH-III reaches full activity at 0.1 ng, and in this respect is 10-fold more potent than angiogenin. At higher doses, ARH-III elicits a maximal response of 41% positive, which is slightly less than that of angiogenin.

Binding of ARH-III to PRI. Competition with RNase A was used to determine that the apparent second-order rate constant, k_a, for the association of ARH-III and PRI is (4.3 ± 0.5) × 10^8 M^{-1} s^{-1}. This value is more than twice that for angiogenin (Table IV).

The dissociation rate constant, k_d, of the ARH-III–PRI complex was determined by measuring the release of free ARH-III from the complex as a function of time. The dissociation rate for the ARH-III–PRI complex is substantially slower than that for angiogenin (Table IV). Over a 10-day period, during which ~8% of the angiogenin–PRI complex dissociated, there was not detectable (<2%) dissociation of the ARH-III–PRI complex (Figure 8). Thus, the upper limit for the k_d for ARH-III can be set conservatively at 3 × 10^{-8} s^{-1}.

The K_i for the interaction of ARH-III and PRI, calculated from k_a and k_d values, is ≤7 × 10^{-17} M. This is at least 10- and 600-fold lower than the values for angiogenin and RNase A, respectively.

Discussion

Angiogenin and RNase A display similarities in amino acid sequence, three-dimensional structure, and capacity to cleave

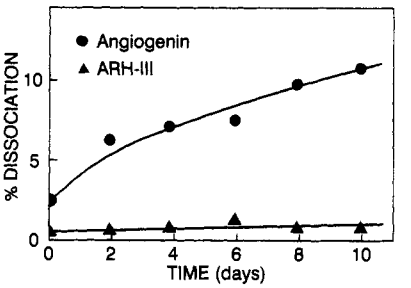

FIGURE 8: Dissociation of angiogenin–PRI and ARH-III–PRI complexes over 10 days. Release of angiogenin and ARH-III was detected by HPLC as described under Experimental Procedures.

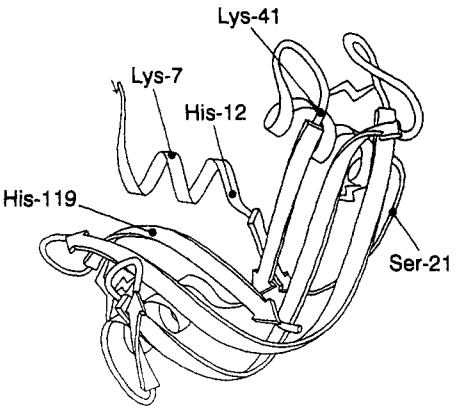

FIGURE 9: Ribbon diagram of RNase A (from J. Richardson). Positions of essential active-site residues His-12, Lys-41, and His-119 are shown, as are the first and last residues of the sequence transferred into ARH-III, Lys-7, and Ser-21.

RNA. Despite this, RNase A is not angiogenic, does not induce a second-messenger response with endothelial cells (Bicknell & Vallee, 1988), and does not display selective cleavage of rRNA in intact ribosomes (St. Clair et al., 1987, 1988). Since the in vivo and in vitro activities of angiogenin clearly differ from those of RNase A, the molecular features which form the bases of these actions should also differ. One approach to identifying such features is to analyze the naturally occurring sequence variation in angiogenins from different species. Regions of the molecule which are critical to its unique activities should be conserved across species, and should also be different from the corresponding regions of the RNases. Once identified, the actual role of these regions can be examined by means of regional mutagenesis.

In an attempt to locate such regions, similarity plots were constructed comparing the sequence of human angiogenin with that of bovine angiogenin (Figure 1a) and RNase A (Figure 1b). The difference between the two plots, 1a minus 1b, was then calculated in order to elucidate those regions of human angiogenin with *both* high identity to bovine angiogenin and low identity to RNase A (Figure 1c). The one segment that stands out is that centered at residues 16–19. Since the analysis employs a five-residue "window", the actual amino acids involved are Tyr-14 through Arg-21. In the three-dimensional structure, these residues (except for Tyr-14) would be in an extended conformation stretching away from the active site, based on comparison to RNase A (Figure 9). Further inspection of this sequence reveals that six of the eight residues are conserved in the mammalian angiogenins (Figure 2a). Only one of them, Asp-15, is also present in RNase A. It is interesting that the corresponding sequence in the pancreatic RNases is very poorly conserved across species. Moreover, residues 15–20 can be removed entirely from RNase A without affecting its enzymatic activity (Hofmann et al.,

1966). Thus, these residues are completely nonessential to RNase A, yet the corresponding sequence in angiogenin is highly conserved.

Immediately preceding residues 14–21 is a block of amino acids from His-8 to His-13 which is conserved in four mammalian angiogenins (Figure 2a); the residues form part of an α-helix which begins at Ser-4, again assuming a similar structure to RNase A (Figure 9). Two residues, His-8 and Thr-11, replace amino acids conserved in 39 mammalian pancreatic RNases, and the acidic Glu-9 of RNase A is replaced by a hydrophobic Leu. Therefore, these residues also show potentially significant differences from RNase A. Accordingly, as a first step toward elucidating the functional role of this entire section of angiogenin, it was decided to investigate residues 8–21, by means of regional mutagenesis. In addition, Asp-22 would also be replaced in order to maintain the original net charge of the protein.

Regional mutagenesis differs from site-directed mutagenesis in that a segment of amino acids, rather than a single residue, is replaced. This enables simultaneous testing of multiple residues, and also allows for examination of secondary structure contributions. Because of the three-dimensional similarities between angiogenin and RNase A, it should be possible to interchange select regions from one protein to the other without disturbing the overall conformation. The efficacy of this approach was demonstrated previously when residues 58–70 of angiogenin were replaced by RNase A residues 59–73 (Harper & Vallee, 1989). The resulting mutant protein, designated ARH-I, folded into the correct conformation and formed not only the three disulfide bonds found in angiogenin but also the fourth disulfide bond from RNase A as well. The amino-terminal region of angiogenin should be equally amenable to replacement, since the peptide Ang-(1–21) has been shown to bind to RNase(21–124) with a micromolar dissociation constant, reconstituting 50% of the enzymatic activity of RNase A (Harper et al., 1988). This demonstrates that there is a structural resemblance between the two proteins in this region.

The amino-terminal region of RNase A has been the subject of numerous crystallographic and chemical studies, due largely to the elaboration of the S-peptide/S-protein[4] system. Residues 3–13 of RNase A form an α-helix (Figure 9) that borders the active-site cleft and contains the catalytic His-12 and substrate-binding Gln-11 residues; the helix binds noncovalently to the S-protein part of the molecule with a submicromolar dissociation constant [see Richards and Wyckoff (1971) and references cited therein]. Residues 15–23 then extend around the outside of the molecule away from the active site, and form a juncture with a second α-helix. Many of the residues have been assigned specific functions [summarized in Richards and Wyckoff (1971, 1973), Blackburn and Moore (1982), and Wlodawer et al. (1982)], including Glu-2 (side-chain H bonds to Arg-10), Phe-8 (hydrophobic interaction with S-protein core), Arg-10 (side-chain H bonds to Glu-2), Gln-11 (side-chain H bonds to substrate phosphate; backbone carbonyl H bonds to Asn-44), His-12 (side-chain involved in catalysis; H bonds to carbonyl of Thr-45), Met-13 (hydrophobic interaction with S-protein core), and Asp-14 (side-chain H bonds to Tyr-25 and/or Arg-33). The importance of these residues is reflected in the fact that, with the exception of Met-13, all are conserved in 39 different mammalian species (Figure 2). In contrast, none of the residues from 15–23 are conserved, and as mentioned, residues 15–20 are completely nonessential.

The sequence of the N-terminal region of angiogenin is shown in Figure 2a; residues conserved in four different species are boxed. Of the first 21 residues, 16 are invariant, and 12 of the 16 are different from their counterparts in RNase A. Several amino acids conserved in all the RNases are replaced in angiogenin; these are Glu-2, Ala-5, Lys-7, and Arg-10. On the basis of comparison with the structure of RNase A, residues 4–14 of angiogenin would form an α-helix with the side chains of Gln-12 and His-13 directed into the active-site cleft. Residues 15–24 would then stretch in a relatively unordered configuration toward the "back" of the molecule, behind several strands of β-sheet, to a juncture with a second α-helix. The region selected for mutation covers residues 8–22 and corresponds to the sequence of RNase A spanning residues Lys-7 to Ser-21 (Figure 9).

Mutation has little effect on the activities of the hybrid protein, ARH-III, toward most RNA substrates. At pH 6.8, ARH-III cleaves tRNA at a rate identical with that of angiogenin (Figure 5a), although there is a slight shift in the pH optimum (Figure 5b). Rates of cleavage of isolated 18S and 28S rRNA (not shown) and CpA, CpG, UpA, and UpG (Table II) are also essentially the same as those for angiogenin. Accordingly, the replaced region does not appear to play a significant role in these activities in angiogenin. Moreover, the relative differences in dinucleotide preference which exist between angiogenin and RNase A (Shapiro et al., 1988; Harper & Vallee, 1989) probably do not stem from substrate–enzyme interactions with any of these residues.

In contrast, the mutation has significant effects on the activities of ARH-III in protein synthesis inhibition and angiogenesis, and also in second-messenger assays. ARH-III is approximately 30-fold less active than angiogenin in inhibiting cell-free protein synthesis; the effects are observed both on the incorporation of [^{35}S]Met into whole protein (Table III) and on the generation of specific rRNA cleavage products from intact ribosomes (Figure 6). In the CAM angiogenesis assay, ARH-III is actually more potent than angiogenin by a factor of 10; it reaches full activity at 0.1 ng per egg whereas angiogenin typically requires 1 ng (Figure 7). Similar results are observed in second-messenger assays with endothelial cells. Dose response curves for the ARH-III-induced release of diacylglycerol or secretion of prostacyclins are shifted to lower concentrations by 10–100-fold compared to those for angiogenin (R. Bicknell, personal communication).

Thus, the mutation only affects angiogenin's unique, non-RNase A type activities—it does not affect general RNA degradation. Since it is precisely these activities which are likely involved in the true biological function of angiogenin, it would appear that the mutated region is at least partly responsible for imparting the molecule's biological action(s). Hence, certain residues and/or structural features in this region are present in angiogenin to fulfill physiological roles not required of their counterparts in the RNases. Such roles might involve binding to a specific ligand or receptor, or perhaps binding and catalysis of a highly specific RNA-containing substrate. The lack of an effect on the general RNase activities of ARH-III does not preclude a role for ribonucleolytic cleavage in angiogenin's mechanism of action, but rather suggests that such cleavage would involve a very specific substrate.

The lack of correlation between the effects of the mutation on angiogenesis (10-fold increase in potency) and those on protein synthesis inhibition (30-fold decrease in potency)

[4] The term S-peptide refers to RNase(1–20), the peptide generated by limited subtilisin proteolysis of RNase A. The remainder of the molecule, RNase(21–124), is referred to as S-protein (Richards & Vithayathil, 1959).

suggests that these two activities are not linked. Similar indications were provided by previous results with ARH-I, which showed an increased capacity to inactivate ribosomes (>10-fold), but a decreased angiogenic activity (Harper & Vallee, 1989). Therefore, these two activities may represent separate, unrelated functions of the angiogenin molecule. The presence of multiple, apparently unrelated activities would not be unique to angiogenin, since this feature is displayed by many cytokines, including basic fibroblast growth factor, transforming growth factor-β, interleukins 1, 2, and 6, and tumor necrosis factor [see Sporn and Roberts, (1988) and references cited therein].

Conversely, the correlation between the effects of the mutation on angiogenesis and second-messenger activity indicates that these two activities of angiogenin may be linked. In both cases, the dose response curve for ARH-III shifts to lower concentrations by 10–100-fold, compared to angiogenin. These data support a growing body of evidence that the linkage between these two activities may involve activation of a putative angiogenin receptor.

ARH-III was also tested for its capacity to interact with PRI, a tight-binding inhibitor of angiogenin and RNases. This 50-kDa protein binds to angiogenin with a K_i of 0.7 fM (Lee et al., 1989b). Interaction minimally encompasses the active site and perturbs the region around Trp-89 (Lee et al., 1989a; Lee & Vallee, 1989a). The interaction of PRI with RNase A, which is 60-fold weaker than that with angiogenin (Lee et al., 1989b), is reported to take place only within the S-protein part of the molecule, with no significant contribution from residues 1–20 (Blackburn & Jailkhani, 1979). It is therefore surprising that mutation of residues 8–22 actually increased the affinity of ARH-III for PRI (Figure 8; Table IV). The association rate more than doubles, and the dissociation rate is decreased to the limit of detection, i.e., at least 4-fold. Consequently, the K_i is at least an order of magnitude lower than that for angiogenin. This change is intriguing in view of the enhanced angiogenic and cellular response activities of ARH-III at low concentrations, and raises the possibility of structural similarities between PRI and a putative angiogenin receptor. The altered binding could result from numerous factors, including more favorable positioning of a PRI contact residue(s), formation of a new ionic interaction, or removal of unfavorable steric or ionic interactions. Lys-8 and Arg-10 of ARH-III would seem to be prime candidates for forming H bonds or ionic interactions with the acidic PRI molecule, since their side chains extend directly out from the active site (based on RNase A structure).

The implication that residues 7–21 of RNase A may also be involved in its interaction with PRI is not consistent with a previous report (Blackburn & Jailkhani, 1979). A change in K_i of 10–100-fold might not have been detected in those experiments, however, as they were based on the assumption that the dissociation rates for the RNase A–PRI and RNase S-protein–PRI complexes are relatively fast ($t_{1/2}$ approximately several minutes). The actual rate for the RNase A–PRI complex, at least, is quite slow ($t_{1/2} \sim 13$ h) (Lee et al., 1989b).

In the present case, as with any investigation involving mutagenesis, the question arises as to the proper refolding of the mutant protein. The evidence for an undistorted conformation for ARH-III is 3-fold: First, all three of the disulfide bonds formed correctly, requiring precise alignment of six cysteine residues from widely separate locations in the sequence. Second, the rates of hydrolysis of four dinucleotides and other RNA substrates are unaffected by the mutation, meaning that positions of the different residues directly involved in phosphodiester bond cleavage were not distorted. Third, ARH-III binds extremely tightly to PRI. This interaction again requires the exact three-dimensional placement of multiple residues which are involved in binding, and the low K_i value ($\leq 7 \times 10^{-17}$ M) is inconsistent with any large alterations.

In summary, comparison of the sequences of angiogenins and pancreatic RNases from different species points to residues 8–21 of angiogenin as being important to its physiological function. Regional mutagenesis was employed to replace this region of angiogenin with the corresponding sequence from RNase A, a homologous protein. The hybrid, ARH-III, shows no change in general ribonucleolytic activities but displays alterations in angiogenic, protein translation inhibition, and second-messenger activities. Thus, the mutation affects primarily those activities which are unique to angiogenin among all proteins in the RNase superfamily, and which are likely involved in its true function(s). Therefore, this region of angiogenin would appear to be involved in its interactions with the specific ligands, receptors, and/or substrates responsible for imparting its biological activities.

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Partial Purification of the 5-Hydroxytryptophan-Reuptake System from Human Blood Platelets Using a Citalopram-Derived Affinity Resin†

E. A. L. Biessen,‡,§ A. S. Horn,§,∥ and G. T. Robillard*,‡
Department of Physical Chemistry and Institute BIOSON and Department of Medicinal Chemistry, Subfaculty of Pharmacy, University of Groningen, Groningen, The Netherlands

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ABSTRACT: This paper describes a procedure for the synthesis and application of a citalopram-derived affinity resin in purifying the 5HT-reuptake system from human blood platelets. A two-step scheme has been developed for partial purification, based on wheat germ agglutinin–lectin (WGA) affinity and citalopram affinity chromatographies. Upon solubilization of the carrier with 1% digitonin, a 50–70-fold increase in specific [^{3}H]imipramine binding activity with a 70% recovery could be accomplished through WGA–lectin chromatography. The WGA pool was then subjected to affinity chromatography on citalopram–agarose. At least 90% of the binding capacity adsorbed to the column. Specific elution using 10 μM citalopram resulted in a 22% recovery of binding activity. A 10 000-fold overall purification was obtained by using this two-step procedure. Analysis of the fractions on SDS–PAGE after ^{125}I labeling revealed specific elution of 78- and 55-kDa proteins concomitant with the appearance of [^{3}H]imipramine binding activity. The pharmacological profile of the partially purified reuptake system correlated well with that derived from the crude membrane-bound reuptake system, suggesting a copurification of the 5HT binding activity and [^{3}H]imipramine binding activity.

The 5HT-reuptake system has been well documented, during the past decades, by both radioligand binding and 5HT-transport studies (Davis, 1984). Substantial evidence has been furnished supporting the concept that the 5HT-reuptake system involves closely interacting sites, a carrier site and a regulatory imipramine binding site (IBS).[1] Binding of reuptake inhibitors to the regulatory site strongly inhibits 5HT reuptake. The structural relationship between the transport and regulatory sites is unclear (Humphreys et al., 1988; Biessen et al., 1988; Reith et al., 1984; Mellerup et al., 1983; Sette et al., 1983). Despite the fact that 5HT reuptake appears to be more or less clarified on a macroscopic level, the molecular basis of transport and its regulation are only poorly understood. The molecular masses of the functional imipramine and paroxetine binding site were estimated by using radiation inactivation studies to be 86 and 67 kDa, respectively (Mellerup et al., 1984). By contrast, photoaffinity labeling of the IBS using [^{3}H]-2-nitroimipramine and [^{3}H]-2-azidoimipramine revealed incorporation of the photoaffinity label in a 30–35-kDa protein (Wennogle et al., 1985; Rotman & Pridluda, 1982). Whether the labeling was specific has not been convincingly demonstrated.

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‡ Department of Physical Chemistry and Institute BIOSON.
§ Department of Medicinal Chemistry, University of Groningen.
∥ A. S. Horn died on January 2, 1990, as a result of injuries sustained in an automobile accident 2 days earlier. Those of us who had the pleasure of knowing him and working with him will miss him dearly.

[1] Abbreviations: AMC, (aminomethyl)citalopram; BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue; Con-A, concanavalin A; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; EI, electron impact; EG, ethylene glycol; IBS, imipramine binding site; KAc, potassium acetate; KP_i, potassium phosphate; NaSCN, sodium thiocyanate; PAA, polyacrylamide; PEG, poly(ethylene glycol); PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; WGA, wheat germ agglutinin.

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