Structural Features That Determine the Enzymatic Potency and Specificity of Human Angiogenin: Threonine-80 and Residues 58−70 and 116−123[†]

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ABSTRACT: Human angiogenin (Ang), a homologue of bovine pancreatic ribonuclease A (RNase A), is a potent inducer of blood vessel formation. It exerts a ribonucleolytic activity that is 10^5−10^6-fold lower than that of RNase A but nonetheless essential for biological action. Previous studies revealed some of the structural features of Ang that underlie its catalytic inefficiency: Gln-117 blocks the space corresponding to the pyrimidine binding site of RNase A and Ang lacks the disulfide loop 65−72 that forms most of the purine binding site of RNase A. Additional features have now been identified by mutagenesis and kinetics. Thr-80, which hydrogen-bonds to the pyrimidine-binding residue Thr-44, plays an important part in attenuating activity and in determining pyrimidine specificity: mutation to Ala increases activity toward cytidylyl substrates by 11−15-fold but has only a minimal effect on cleavage of uridylyl substrates. The properties of T44A/T80A and Q117A/T80A double mutants demonstrate that these changes are mediated by Thr-44 and are largely independent of the blockage by Gln-117. The side chain of Ser-118 also suppresses enzymatic activity: S118A is 5−7-fold more effective than Ang. This increase appears to reflect the loss of a hydrogen bond with Asp-116 that helps to orient Gln-117. The effects of deleting residues 119−123 suggest that main-chain atoms of the C-terminal 3^{10} helix make a small further contribution. Finally, the significance of the absence of the RNase A loop 65−72 from Ang has been investigated by reexamining the earlier derivative ARH-I (in which Ang residues 58−70 have been replaced by residues 59−73 of RNase) and generating new derivatives of this hybrid protein. The results suggest that the RNase A segment of ARH-I not only provides more effective purine recognition but also counteracts the deleterious effects of Gln-117 and Thr-80 on the pyrimidine site.

Several proteins in the pancreatic ribonuclease superfamily have been demonstrated to be potent biological effectors (see ref *1* for review): angiogenin (Ang)[1] is an inducer of new blood vessel formation (*2*, *3*) that appears to be critically involved in tumorigenesis (*4*, *5*); bovine seminal RNase (BS-RNase) (*6*, *7*) and the frog RNase onconase (*8*) inhibit tumor growth, and BS-RNase is immunosuppressive as well (*9*); both eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) are toxic to neurons and certain parasites (*10*−*12*), and ECP has antibacterial activity (*13*). In all but one instance—the antibacterial effect of ECP (*14*)—the physiological activities of these RNases require their enzymatic action (*15*−*21*). The evidence for this association is particularly extensive with Ang. Mutations of any of the three catalytic residues His-13, Lys-40, and His-114 in human Ang essentially abolish both activities (*15*,*16*). Replacement of the substrate binding residue Thr-44 by His also attenuates both activities in parallel, whereas substitution by Asp does not diminish activity toward RNA and has no impact on angiogenicity (*17*). Moreover, an oligonucleotide inhibitor of the enzymatic activity of Ang reduces angiogenic activity as well (V. Nobile and G.-f. Hu, personal communication).

Ang is unique among the RNases with respect to both its mode of action and its enzymatic properties. The others exert toxic effects that in many cases are undoubtedly the consequence of cellular uptake followed by cytosolic RNA degradation [e.g., see ref *22*]. Ang, on the other hand, is a stimulatory agent that elicits endothelial cell proliferation and migration (*23*, *24*) culminating in the formation of a new hemovascular network. Although the precise mechanism by which Ang utilizes its enzymatic activity to achieve this has not been determined (see Discussion), it seems unlikely that general destruction of intracellular RNA components is involved. Ang is also distinguished by its extremely weak ribonucleolytic activity toward standard types of RNase substrates (*25*). Thus, BS-RNase (*26*, *27*) and EDN (*28*−*30*) are virtually equivalent to bovine pancreatic RNase A with high molecular weight RNA substrates, whereas ECP (*28*, *31*) and onconase (*32*) are ∼1% as active. In contrast,

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[1] Abbreviations: Ang, angiogenin; BS-RNase, bovine seminal ribonuclease; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin (also known as RNase U_s, liver RNase, and placental RNase); ARH-I, angiogenin/ribonuclease A hybrid protein in which angiogenin residues 58−70 have been replaced by amino acids 59−73 of ribonuclease A; ppA-2′-p, 5′-diphosphoadenosine 2′-phosphate; HPLC, high-performance liquid chromatography; C18, octadecylsilane; C>p, cytidine cyclic 2′,3′-phosphate.

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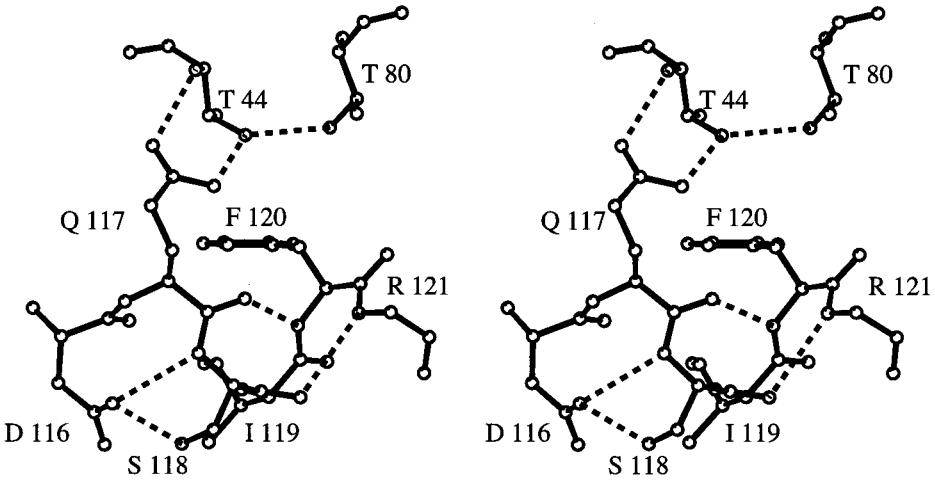

FIGURE 1: Stereoview of the C-terminal residues 116−121 and the putative B_1 subsite of human Ang, showing hydrogen bonds (distances are listed in Table 1). The side chain of Arg-121 has been omitted for clarity. Atomic coordinates are from the crystal structure of Acharya et al. (*40*), extended to 2.0 Å resolution (D. D. Leonidas and K. R. Acharya, personal communication). The figure was drawn with the program MOLSCRIPT (*83*).

Ang is nearly 5 orders of magnitude less potent toward RNA than RNase A, and with some small substrates the difference is more than a factor of 10^6 (*33*−*35*). The low catalytic efficiency of Ang reflects both its relatively slow turnover rate and its weak affinity for nucleotides: k_{cat} and K_m values for the best dinucleotide substrate, CpA, are 0.7 s^{-1} and 62 mM, and the K_i value for 2′-CMP is 9 mM, i.e., 3000-fold higher than that measured with RNase A (*36, 37*). Thus, the Ang/RNase system offers an unusual opportunity to utilize site-directed mutagenesis to *gain*, rather than lose, function, i.e., by replacing the residues responsible for maintaining the low RNase activity of Ang. This approach may provide important insights into the structural basis for Ang's enzymatic weakness that can be exploited for the design of Ang antagonists as potential antitumor drugs.

The active site of RNase A is generally described in terms of various subsites (*38, 39*): these include a P_1 site at which phosphodiester bond cleavage occurs, a B_1 site for binding the pyrimidine base whose ribose donates its 3′-oxygen to the phosphate in P_1, and a B_2 site that preferentially binds a purine ring on the opposite side of the scissile bond. Many of the major functional components of RNase A are conserved in the Ang sequence, among them the catalytic triad His-12, Lys-41, and His-119 (His-13, Lys-40, and His-114 in Ang) and B_1-site residues Thr-45 and Ser-123 (Thr-44 and Ser-118 in Ang). In contrast, most of the B_2 site of RNase A—that contributed by the 65−72 disulfide loop—is not maintained in Ang, which lacks the disulfide and has an entirely distinct sequence in this region. The introduction of the RNase segment containing this loop into Ang by mutagenesis, producing the Ang−RNase A hybrid protein ARH-I, enhances enzymatic activity by up to several hundred-fold (*34*). Thus, differences between Ang and RNase A in this segment are an important factor in determining their relative potencies.

The crystal structure of Ang (*40*) has revealed a completely unsuspected additional feature of the protein that attenuates its enzymatic activity: the site that is spatially analogous to the B_1 site of RNase A is blocked by Gln-117. This obstruction also exists in solution (36) and thus it can be inferred that Ang must undergo a structural reorientation in order to bind and cleave RNA substrates. The native conformation of Ang, which is presumably catalytically inactive, appears to be stabilized by multiple hydrogen bonds (Figure 1 and Table 1) and the hydrophobic effect. The side chain of Gln-117 forms two hydrogen bonds with Thr-44 that mimic those made by pyrimidines with Thr-45 of RNase A (*41, 42*). Thr-44 in turn hydrogen-bonds to Thr-80 on the side opposite Gln-117. The residues that flank the glutamine, Asp-116 and Ser-118, interact with each other via two hydrogen bonds. In addition, main-chain interactions connect Gln-117 with Phe-120 and Ser-118 with Arg-121 in a C-terminal 3^{10} helix that is absent from RNase A. Finally, the side chains of Ile-119 and Phe-120 are largely buried in a pocket lined by the nonpolar residues Val-78, Val-103, Val-104, and Leu-115.

Table 1: Hydrogen Bonds in the C-Terminal Region and Putative B_1 Site of Angiogenin[a]

donor	acceptor	distance (Å)
Thr-44 N	Gln-117 OE1	2.9
Thr-44 OG1	Thr-80 OG1	2.7
Gln-117 NE2	Thr-44 OG1	3.1
Ser-118 OG	Asp-116 OD2	2.6
Ser-118 N	Asp-116 OD2	3.0
Phe-120 N	Gln-117 O	3.0
Arg-121 N	Ser-118 O	3.2

[a] See Figure 1.

Previous mutational studies have examined the roles of some of the amino acids that contribute to this unusual active-site architecture. Replacements of Gln-117 increase enzymatic activity by as much as 30-fold (*36*), consistent with the obstructive position of this residue in the crystal structure. Mutations of Asp-116 amplify activity by up to 18-fold (*43*), an originally inexplicable result that can now be understood in terms of the loss of hydrogen bonds that help maintain the adverse orientation of Gln-117. Simultaneous replacements of Ile-119 and Phe-120 by Ala produce a 4-fold enhancement, again in agreement with structural observations (*44*).

In the present study, additional residues and interactions in this region of Ang have been investigated by mutagenesis and kinetics. The results demonstrate that Thr-80 plays a major part in attenuating the enzymatic activity of Ang and

in determining pyrimidine specificity; surprisingly, the mechanism by which it suppresses catalytic potency appears to be largely independent of the B_1-site blockage by Gln-117. The roles of the Ser-118 side chain and main-chain atoms of the 3^{10} helix have also been evaluated. Finally, the basis for the markedly increased activity of ARH-I has been reexamined. The kinetic properties of ARH-I and its derivatives suggest that the RNase A segment of this hybrid protein not only provides more effective base recognition at the B_2 site but also serves to counteract the deleterious effects of Gln-117 and Thr-80 on the B_1 site.

EXPERIMENTAL PROCEDURES

The natural <Glu-1 form of human Ang was obtained from a recombinant system in *Escherichia coli* (*33*). The plasmid pAng3, which contains a synthetic gene for Ang appended to the *E. coli phoA* signal peptide sequence, was from an earlier study (*45*). The expression plasmid for the Ang−RNase hybrid protein <Glu-1 ARH-I was generated from an earlier construct for Met-(−1) ARH-I (*34*) by ligating the *Xho*I/*Eco*RI fragment of the gene (*33*) into pAng3 that had been digested with the same enzymes. Angiogenin substrates and inhibitors were purchased from Sigma, with the exception of 5′-diphosphoadenosine 2′-phosphate (ppA-2′-p), which was from an earlier study (*37*). Oligonucleotides were obtained from Promega (Madison) or Amitof (Boston). Three-dimensional structures of Ang and RNase A were examined and superimposed with the program QUANTA (Molecular Simulations), implemented on a Silicon Graphics Indy computer.

Oligonucleotide-Directed Mutagenesis. Genes encoding angiogenin variants were prepared by PCR except as noted below. The templates for PCR were pAng 3 [for generation of T80A, T80D, S118A, D116A/S118A, and des(119−123)], the mutant derivative of pAng3 encoding T80A (for T44A/T80A,Q117A/T80A), and the plasmid encoding ARH-I described above (for T80A/ARH-I). Mutations of residues in the C-terminal segment 116−123 were introduced by amplifying the portion of the gene encoding residues 58−123. The 5′ primer used in all cases and the 3′ primer for mutation of Gln-117 were those reported by Russo et al. (*36*). The other mutagenic primers were 5′-TTAAGGCCTTCG-GAAGATAGCCTGATCTAGATG-3′ (S118A), 5′-TTAAGG-CCTTCGGAAGATAGCCTGAGCTAGATGGACTGG-3′ (D116A/S118A), and 5′-TTAAGGCCTTCGGAATTAAGA-CTGATCTAGATG-3′ [des(119−123)]. PCR products were cleaved and ligated into pAng3 as detailed previously (*36*). Plasmids encoding T80A, T80D, T44A/T80A, and ARH-I/T80A were constructed by PCR overlap extension (*46*). The complementary mutagenic primers were 5′-CCAGGTTAC-AGCTTGCAAACTTC-3′ and 5′-AAGTTTGCAAGCTGT-AACCTGGA-3′ (T80A), 5′-TCCAGGTTACAGATTGCA-AACTTC-3′ and 5′-AGTTTGCAATCTGTAACCTGGAAG-3′ (T80D), and 5′-AAGATATCAACGCTTTCATCCATG-3′ and 5′-TGGATGAAAGCGTTGATATCTTTG-3′ (T44A). The 5′- and 3′-flanking primers and details of the method were as described (*47*). The plasmid for expression of Q117A/ARH-I was generated by using a Quick Change kit (Stratagene) according to the manufacturer's recommendations. The template was the plasmid for <Glu-1 ARH-I, produced in *E. coli* strain W3110, and the mutagenic primers were 5′-GTCCATCTAGATGCGTCTATCTTCCGACTT-CCTTAATAG-3′ and 5′-CTATTAAGGCCGTCGGAAGAT-AGACGCATCTAGATGGAC-3′. These primers introduce an additional, silent mutation that eliminates the *Stu*I site and thereby facilitates screening of transformants. The entire coding region of the Q117A/ARH-I plasmid and the PCR-derived portions of the plasmids for the other mutants were sequenced to establish the presence of the desired mutations and rule out any spurious changes.

Expression, Isolation, and Structural Characterization of Variant Proteins. Ang variants were expressed and purified to homogeneity as described (*45*). The properties of all derivatives during Mono S cation-exchange chromatography and C18 HPLC were consistent with their proposed structures. The Mono S retention times of S118A, T80A, Q117A/T80A, and T44A/T80A were indistinguishable from those of Ang; D116A/S118A and the ARH-I derivatives, whose net charges are 1 and 2 units, respectively, more positive than that of Ang, eluted 2−4 min later; T80D and des(119−123), whose net charges are 1 and 2 units, respectively, less positive than that of Ang, eluted 2−3 min earlier. The C18 HPLC retention times of des(119−123) and the ARH-I double mutants were 1−2 min shorter than for Ang, D116A/S118A eluted 3 min later than Ang, and the others eluted at virtually the same time as Ang. The amino acid compositions of all variant proteins confirmed the presence of the intended replacement(s) and showed no other significant changes (data not shown). These compositions, together with SDS−PAGE and C18 HPLC results, indicated that all products were >95% pure.

Enzymatic Assays. Preparations of Ang and its derivatives used for kinetic determinations were quantitated by amino acid analysis. Ribonucleolytic activity toward tRNA was measured with a precipitation assay (*48*). Incubations were performed in 33 mM Hepes/33 mM NaCl, pH 6.8, for 2 h at 37 °C. Activity was quantitated by comparison with a standard curve obtained with 0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 μg of unmodified Ang. The dependence of ΔA_{260} on enzyme concentration [E] in this assay system is nonlinear for Ang (see ref *33*). The shape of the ΔA_{260} vs [E] plot was similarly nonlinear for all Ang variants examined except for ARH-I (34), ARH-I/T80A, and ARH-I/Q117A, which produced nearly linear plots. Because of this difference in shape, activities for ARH-I and its derivatives relative to Ang depended on which portion of the Ang standard curve was used; the activities reported were calculated on the basis of the ΔA_{260} values produced by 0.8−1.6 μg of Ang.

Values for k_{cat}/K_m with dinucleotide substrates and C>p were determined by a modification (*36*) of the method of Shapiro et al. (*49*). Briefly, incubations were performed with 100 μM substrate in 0.2 M Mes, pH 5.9, at 25 °C; reaction mixtures that contained <0.3 μM enzyme were supplemented with 0.1 mg/mL RNase-free bovine serum albumin (Worthington). After various times, reaction mixtures were analyzed by C18 HPLC (*36*), and k_{cat}/K_m values were calculated from the extent of substrate cleavage as described (*49*). The substrate concentration used is expected to be well below K_m for all Ang variants since (i) this concentration is ~600-fold and 5-fold lower than the K_m values for Ang and RNase A, respectively, with CpA, the best dinucleotide substrate for both enzymes (*36*), and (ii) the decrease in substrate concentration with time is first-order in all cases.

Inhibition by 2′-CMP, 5′-AMP, and ppA-2′-p was assessed

Table 2: Relative Activities of Angiogenin and Angiogenin Variants toward tRNA[a]

enzyme	rel activity	enzyme	rel activity
Ang	1.00	S118A	7.1
T80A	10.1	D116A[b]	15
T44A[b]	0.04	D116A/S118A	24
T44A/T80A	0.02	des(119−123)	1.2
Q117A	14	ARH-I	410
Q117A/T80A	83	Q117A/ARH-I	600
T80D	2.8	T80A/ARH-I	520

[a] Activities were determined as described under Experimental Procedures. [b] Values for T44A and D116A are from refs *17* and *43*, respectively; activities listed for the other previously examined angiogenin variants, Q117A and ARH-I, were measured at the same time as for the new derivatives.

by measuring their effects on k_{cat}/K_m values for cleavage of CpA. With 2′-CMP, reaction products were analyzed by C18 HPLC as described (*36*). With the other inhibitors, products were quantitated by Mono Q chromatography with a 15-min linear gradient from 0 to 0.15 M NaCl in 10 mM Tris, pH 8, at a flow rate of 1 mL/min. Effluents were monitored at 254 nm. K_i values were calculated by performing nonlinear fits of the data to the equation $(k_{cat}/K_m)_i = (k_{cat}/K_m)_0/(1 + [I]/K_i)$ with SigmaPlot 4.0 (SPSS Inc., Chicago), where $(k_{cat}/K_m)_i$ and $(k_{cat}/K_m)_0$ are values in the presence and absence of inhibitor, respectively, and [I] is inhibitor concentration. Four or five inhibitor concentrations were employed, and each assay was performed in duplicate.

RESULTS

Effects of Thr-80 Replacements. In the Ang crystal structure, the side-chain OH of Thr-80 hydrogen-bonds with that of Thr-44 (Figure 1). Thr-44 is a key functional component of the B_1 site: replacements by Ala, His, and Asp markedly alter pyrimidine specificity, with the first two substantially diminishing activity toward RNA (*17*; data for T44A are shown in Tables 2 and 3). The substrate-binding role of Thr-44 is not evident from the native structure, in which this residue is inaccessible owing to the position of Gln-117, and presumably is fulfilled only after the enzyme has undergone a transition to its "active conformation".

The functional significance of the 44/80 hydrogen bond was investigated initially by mutating Thr-80 to Ala. This replacement dramatically enhances ribonucleolytic activity toward tRNA, CpN dinucleotides, and the cyclic 2′,3′ nucleotide C>p, by factors ranging from 10 to 15 (Tables 2 and 3). In contrast, the k_{cat}/K_m value for cleavage of UpA is essentially unchanged (1.3 vs 1.2 M^{-1} s^{-1}), and that for UpG is increased by just 2-fold (from 0.28 to 0.55 M^{-1} s^{-1}; not listed in table). As a consequence, T80A exhibits a much stronger preference for cytidine vs uridine dinucleotides than does Ang, i.e., ∼120-fold compared to 12−16-fold. The improvement in activity toward cytidylyl substrates by T80A is not paralleled by any appreciable increase in binding affinity: the K_i value for 2′-CMP is only 2-fold lower with the variant protein than with Ang (Table 4).

The structural basis for the enhanced potency of T80A was examined by individually replacing Thr-44 and Gln-117 in this derivative with Ala (Tables 2 and 3). The Thr-44 substitution completely nullifies the activity increase. Indeed, T44A/T80A has even lower activity toward tRNA and CpA than does Ang and is functionally similar to the single-residue mutant T44A. Thus, Thr-44 mediates the effects of the Thr-80 substitution. Replacement of Gln-117 by Ala in T80A increases activity by 4−12-fold, depending on substrate, comparable to the changes produced by this mutation in Ang (6−16-fold). Q117A/T80A is 60−132-fold more active than Ang with tRNA and cytidylyl substrates and retains the strong preference of T80A for CpN vs UpN dinucleotides.

Asp-83 of RNase A corresponds to Thr-80 of Ang. It too interacts with the B_1-site threonine (Thr-45) (*50*) and influences pyrimidine specificity, although positively rather than negatively (*51*). Functional differences between the Ang and RNase A residues were explored by replacing Thr-80 of Ang with Asp. The k_{cat}/K_m values for T80D with CpA, CpG, CpC, and C>p are all somewhat lower than those of Ang (Table 3); i.e., an Asp in this position suppresses activity

Table 3: Enzymatic Activities of Angiogenin and Angiogenin Variants toward CpA, CpG, UpA, CpC, and C>p[a]

	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)[b]				
enzyme	CpA	UpA	CpG	CpC	C>p
Ang[c]	14.6 ± 0.3 (**1.0**)	1.2 ± 0.2 (**1.0**)	4.5 ± 0.1 (**1.0**)	1.4 ± 0.4 (**1.0**)	0.96 ± 0.04 (**1.0**)
T80A	166 ± 15 (**11.4**)	1.3 ± 0.1 (**1.1**)	66 ± 6 (**14.7**)	18.7 ± 0.1 (**13.4**)	13.5 ± 0.4 (**14.1**)
T44A[d]	0.5 (**0.04**)	0.4 (**0.4**)			
T44A/T80A	0.33 ± 0.01 (**0.02**)	0.41 ± 0.05 (**0.34**)			
Q117A[c]	148 ± 2 (**10.1**)	7.5 ± 0.6 (**6.3**)	47 ± 1 (**10.4**)	22.6 ± 1.5 (**16.1**)	11.7 ± 0.4 (**12.2**)
Q117A/T80A	1920 ± 100 (**132**)	12.6 ± 0.7 (**10.5**)	270 ± 37 (**60**)	91 ± 9 (**65**)	77 ± 4 (**80**)
T80D	10.3 ± 0.4 (**0.71**)	20.4 ± 0.5 (**17**)	3.5 ± 0.1 (**0.78**)	1.2 ± 0.1 (**0.9**)	0.45 ± 0.05 (**0.47**)
S118A	93 ± 1 (**6.4**)	5.3 ± 0.3 (**4.4**)	25.6 ± 1.2 (**5.7**)	8.8 ± 0.3 (**6.3**)	4.5 ± 0.4 (**4.7**)
D116A[d]	20 (**1.7**)	0.9 (**0.9**)			
D116A/S118A	52 ± 2 (**3.6**)	5.2 ± 0.2 (**4.3**)			
des(119−123)	102 ± 4 (**7.0**)	5.2 ± 0.2 (**4.3**)	23.8 ± 1.7 (**5.3**)	8.9 ± 0.2 (**6.4**)	5.1 ± 0.2 (**5.3**)
ARH-I[c]	1230 ± 50 (**84**)	370 ± 5 (**308**)	43 ± 7 (**9.6**)		24.5 ± 0.9 (**25.5**)
Q117A/ARH-I	3670 ± 180 (**251**)	1900 ± 80 (**1580**)	246 ± 31 (**55**)		73 ± 12 (**76**)
T80A/ARH-I	2490 ± 220 (**171**)	290 ± 11 (**242**)	196 ± 3 (**44**)		54 ± 9 (**56**)

[a] Activities were determined as described under Experimental Procedures. [b] Values for k_{cat}/K_m are mean ± SD ($n = 3$). Values relative to those with unmutated Ang are shown in boldface type in parentheses. [c] Values for Ang, Q117A, and ARH-I were measured at the same time as for the new variants. Activities of Q117A toward CpC and C<p and of ARH-I toward C>p were not reported previously. Other activities shown for Ang, Q117A, and ARH-I differ somewhat from those determined in earlier studies (*34, 36, 37*). [d] Values listed for D116A and T44A are from refs *43* and *17*, respectively; the conditions for assaying activity toward dinucleotides differed from those used here and yielded k_{cat}/K_m values of 12, 4, and 1 M^{-1} s^{-1} for Ang with CpA, CpG, and UpA, respectively. The latter values were used for calculating the relative activities shown for these derivatives.

Table 4: Inhibition of Ang, ARH-I, and T80A by Various Nucleotides[a]

	K_i (mM)		
enzyme	2′-CMP	5′-AMP	ppA-2′-p
Ang	5.4	4.7	0.11
T80A	2.6		
ARH-I	0.55	0.59	0.009

[a] K_i values were determined as described under Experimental Procedures.

toward cytidylyl substrates even more effectively than a Thr. However, T80D has 17-fold greater activity than Ang toward UpA and, as a result, its pyrimidine preference (U > C) is opposite to that of Ang. T80D degrades tRNA 2.8-fold more rapidly than does Ang (Table 2), likely reflecting its increased action at uridylyl nucleotides.

Effects of Mutations in the C-Terminal Segment. The results of earlier mutational studies have revealed the contributions of the side chains of Asp-116, Gln-117, Ile-119, and Phe-120 in stabilizing the blockage of the B_1 site of Ang (*36*, *43*, *44*, *52*). Two other C-terminal components that appear to participate in defining the inactive native conformation—i.e., the side chain of Ser-118 and main-chain atoms of the 116−121 3^{10} helix (Figure 1 and Table 1)—have now been investigated. Replacement of Ser-118 by Ala enhances activity toward tRNA by 7-fold; a 2.1-fold larger increase accompanies substitution of Asp-116, the hydrogen-bonding partner of this serine, by Ala (*43*) (Table 2). The k_{cat}/K_m values of S118A for cleavage of the dinucleotides CpA, CpG, UpA, and CpC and hydrolysis of C>p are increased by 4.7−6.4-fold, in contrast with the minimal impact of the Asp-116 mutation on activity toward CpA and UpA (Table 3). The relationship between Asp-116 and Ser-118 was studied further by producing and characterizing the double mutant D116A/S118A. This derivative has 25-fold greater activity toward tRNA than does Ang: i.e., it is more effective than either single-residue mutant (Table 2). With dinucleotides, the potency of D116A/S118A is higher than that of D116A but equivalent to (UpA) or somewhat lower than (CpA) that of S118A (Table 3).

The contribution of the two backbone hydrogen bonds in the C-terminal 3^{10} helix (Gln-117 to Phe-120 and Ser-118 to Arg-121; Table 1 and Figure 1) to activity was assessed by deleting the last five residues of the protein, 119−123. Removal of only 120−123 would have been sufficient to eliminate the interactions of interest, but the deletion was extended to Ile-119 to minimize potential interpretive ambiguities: the 119 side chain in Ang is partially buried through its contacts with Phe-120 (*40*, *44*), and might become exposed to solvent in a des(120−123) derivative. Des(119−123) Ang exhibits 4.3−7-fold increased activity toward small substrates (Table 3) but is only slightly more active than Ang with tRNA (Table 2). The implications of these results for the role of main-chain interactions are addressed in the Discussion.

Effects of Replacement of the Segment 58−70: ARH-I Revisited. The original study on ARH-I (*34*), an Ang−RNase A hybrid protein in which RNase A residues 59−73 replace the corresponding residues (58−70) of Ang, was performed prior to the determination of the three-dimensional structure of Ang. At that time, the substantially increased enzymatic activity of ARH-I was attributed to improved purine recognition at the B_2 site and enhanced reactivity of the catalytic histidines and lysine, which were shown to undergo chemical modification more rapidly in this derivative than in Ang. The basis for the enhanced activity of ARH-I is now reconsidered in light of the unexpected obstruction of the Ang active site observed in the crystal structure. Superposition of the atomic coordinates for Ang and RNase A (*40*, *53*) reveals that the Ser-118 side chain of Ang and the CE-NZ portion of Lys-66 of RNase fill essentially identical spaces (Figure 2), suggesting that the introduction of the RNase A segment into Ang might facilitate or even induce the rearrangement of the C-terminal region that opens the B_1 site. This possibility was examined by performing additional kinetic measurements with ARH-I and by generating the derivative Q117A/ARH-I.

The K_i value for binding of the pyrimidine nucleotide 2′-CMP to ARH-I was found to be 10-fold lower than with Ang (Table 4). This improvement in affinity is similar to that measured with the purine nucleotides 5′-AMP (8-fold) and ppA-2′-p (12-fold), suggesting that the ARH-I regional replacement indeed affects the B_1 site as well as B_2. Consistent with this view, the k_{cat}/K_m value for hydrolysis

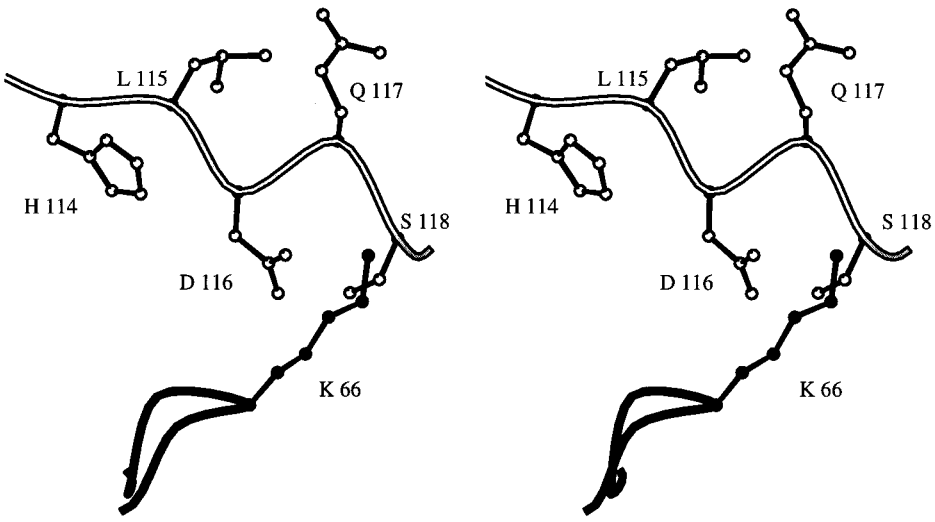

FIGURE 2: Stereoview of a superposition of the crystal structures of Ang (as in Figure 1) and RNase A (*53*), showing residues 114−118 of Ang (open) and Lys-66 plus the 63−69 main chain of RNase A (solid). The figure was drawn with the program MOLSCRIPT (*83*).

of C>p, a substrate expected to occupy B_1 but not B_2, is 25-fold higher with ARH-I than with Ang (Table 3). The impact of the mutation on B_1 was probed further by substituting Ala for Gln-117 in ARH-I (Ang numbering); replacement of Gln-117 in Ang has been shown to influence the B_1 subsite but not B_2 or P_1 (*36*). The activity of Q117A/ARH-I toward tRNA is only 50% higher than that of ARH-I, i.e., 600× that of Ang vs 410× (Table 2), indicating that the effects of the two mutations on cleavage of this polynucleotide substrate are not independent. Larger increases, ranging from 3- to 6-fold, are observed with dinucleotide and cyclic nucleotide substrates (Table 3).

The impact of the ARH-I replacement on the suppressive role of Thr-80 was also explored, by producing the derivative T80A/ARH-I. The activity of this derivative toward tRNA is only 26% higher than that of ARH-I and values for k_{cat}/K_m with CpA, CpG, and C>p are increased by 2−3-fold compared to those of ARH-I, in all cases well below the factor of 11−15 anticipated if the two mutations exert their effects through fully distinct mechanisms. T80A/ARH-I is slightly less active than ARH-I toward UpA.

DISCUSSION

Ang contains the four RNase A residues that are most critical for enzymatic activity: the three that comprise the actual catalytic apparatus (His-13, Lys-40, and His-114) and the pyrimidine-binding component Thr-44 (Ang numbering). Although there are subtle differences between the positions of these amino acids in the Ang [1ANG (*40*); Brookhaven Protein Data Bank accession number] and RNase A three-dimensional structures [e.g., 7RSA (*53*), 3RN3 (*54*), and 1AFU (*55*)], these deviations are not substantially greater than those observed among various RNase A structures and are unlikely to account for any major disparities in enzymatic capacities by themselves. However, the effectiveness of these residues is greatly curtailed by other active-site substituents. On one side of Thr-44 in the free Ang structure, Gln-117 occupies the space that must ultimately be filled by the substrate pyrimidine moiety in order for His-13, Lys-40, and His-114 to serve their catalytic functions. On the opposite side, Thr-44 forms a hydrogen bond with Thr-80 which has here been demonstrated to impair function. The present findings indicate that both the B_1 site blockage by Gln-117 and the activity-suppressing role of Thr-80 are intimately connected with the absence from Ang of a short disulfide loop present in all other known mammalian RNases. These and additional factors contributing to the attenuation of angiogenin's ribonucleolytic activity toward typical RNase substrates are now considered in detail.

Blockage of the B_1 Site. The obstruction of B_1 by Gln-117 is the most striking and unique characteristic of the native Ang structure. RNase A and all of the additional homologues whose three-dimensional structures have been determined—EDN (*56*), BS-RNase (*57*), and onconase (*58*)—have open B_1 sites. X-ray diffraction (*59*) and NMR (*60*) studies of bovine Ang reveal the same blockage as in the human protein, but by a glutamic acid (118) rather than a glutamine. Mouse, rabbit, and pig angiogenins also contain Glu at this position (*61*) and are predicted to have similar structures. Molecular modeling indicates that RNA substrates cannot bind productively to the native Ang structure, no matter what conformation they adopt; since Ang nonetheless cleaves these molecules, it has been postulated that the protein itself undergoes a conformational transition that removes the Gln/Glu impediment as part of its normal catalytic pathway (*36, 40*). The rearrangement of Ang may occur prior to substrate binding—i.e., a major "B_1 closed" form may be in equilibrium with a minor "B_1 open" structure—or it may be induced by interaction with the substrate. In either case, the energetic cost of opening the cryptic site results in diminished catalytic efficiency.

The positioning of Gln-117 within the putative B_1 site of human Ang appears to be accomplished by a combination of factors (Figure 1; *40*). The secondary structure of the C-terminal segment of Ang starting with Asp-116 is helical, whereas its counterpart in RNase A is a β-strand; the first turn of the helix places Gln-117 next to Thr-44, whereas the β-strand of RNase directs the corresponding residue (Ala-122) away from the B_1 site. The Ang helix also juxtaposes Asp-116 and Ser-118, which form two hydrogen bonds. The counterparts of these amino acids in RNase A (Asp-121 and Ser-123, respectively) are oriented quite differently and make positive contributions to activity. The side chain of Asp-121 is shifted ∼3 Å from the position of Asp-116 in Ang and hydrogen-bonds to the catalytic residue His-119; replacements of Asp-121 in RNase diminish activity by 10−40-fold (*62*). The Ser-123 hydroxyl is even farther (∼7 Å) from that of Ser-118 in Ang and plays a minor role in pyrimidine binding (*63*) through a water-mediated hydrogen bond (*64*). The overall orientation of the helical segment in Ang seems to be fixed in part by burying the Ile-119 and Phe-120 side chains against those of hydrophobic residues in β-strands 4, 6, and 7 (*40*).

Earlier mutagenesis results support the existence of the activity-suppressing roles for Gln-117 and Asp-116 seen in the Ang crystal structure. Mutations of Gln-117 by Ala and Gly enhance enzymatic potency by as much as 18-fold and 30-fold, respectively (*36*). Modeling shows that truncation of the Gln side chain by itself would not open the B_1 site sufficiently to permit pyrimidine binding (*36*); recent X-ray diffraction data on Q117G confirm this and reveal that the C-terminal conformation is unchanged (D. D. Leonidas and K. R. Acharya, personal communication). Thus, the interactions of other residues (see Figure 1) appear to be sufficient to prevent reorientation of this segment. Replacements of Asp-116 by His, Ala, and Asn augment activity toward tRNA by 18-, 15-, and 9-fold, respectively (*43*). Several other substitutions of this residue also produce improvements, and only the structurally disruptive mutations to Pro and Trp have negative effects (*52*). At the same time, replacements of Ser-118, the hydrogen-bonding partner of Asp-116, by Arg and Asp diminish activity by 2.5- and 4-fold, respectively (*17*) although in both cases the side-chain interaction with Asp-116 has been altered or abolished.

In the present study, the role of Ser-118 was examined by Ala replacement, and the marked increase in activity anticipated from the three-dimensional structure was indeed obtained. The extent of the improvement with tRNA as substrate is about half of that achieved by Ala substitution of Asp-116, a result that is not surprising in view of the fact that only one of the two 116/118 hydrogen bonds is eliminated directly by mutating the Ser, whereas replacing the Asp removes both (Figure 1 and Table 1). An alternative

explanation for the less substantial effect of 118 vs 116 substitution—that it might indicate that Ser-118 exerts a positive influence on activity in addition to its negative one—is ruled out by the increased effectiveness of the double 116/118 Ala mutant compared to the single-residue derivatives. Moreover, this finding suggests that one or both of these residues may constrain activity by some additional means not apparent from the crystal structure. A complete picture of the roles of Asp-116 and Ser-118 must ultimately account for another puzzling observation as well: replacement of Asp-116 barely alters activity toward small substrates, whereas replacement of Ser-118 enhances cleavage of dinucleotides almost as much as it does that of polynucleotide substrates (Tables 2 and 3).

The importance of backbone hydrogen bonds within the C-terminal helix for stabilizing the native obstructive conformation of Ang was investigated by producing the des(119−123) derivative. Although the interpretation of the effects of this mutation is complicated by the simultaneous elimination of side-chain interactions, prior data on I119A/F120A and des(121−123) derivatives (*44*) allow some conclusions to be drawn. I119A/F120A has 3−4-fold increased activity toward all substrates examined, consistent with the proposed role of the 119 and 120 side chains. Des(121−123) has 10-fold lower activity than Ang with polynucleotides but is only slightly less effective than Ang with dinucleotides and C>p. This suggests that residues 121−123 do not contribute to the inactive C-terminal structure and instead constitute a peripheral subsite for binding larger substrates. The present 119−123 deletion incorporates the combined structural changes of the I119A/F120A and des(121−123) mutations, plus the removal of the 119/120 main chain and β-methyl groups. Des(119−123) is virtually equivalent to Ang with tRNA as substrate and 4−7-fold more active toward dinucleotides. These activities are a few-fold higher than would be anticipated from the combination of the two earlier mutations, suggesting that the 119/120 backbone may make a small but significant contribution to stabilizing the native conformation of Ang. The marked difference in the consequences of the deletion for cleavage of large vs small substrates in des(119−123) is consistent with the proposed function of the segment 121−123 in recognition of peripheral RNA components (*44*).

Role of Thr-80. The Ang crystal structure suggests two antithetical potential functions for the hydrogen bond between the side chains of Thr-44 and Thr-80: a negative role as part of a network maintaining the native obstructive conformation, and a positive one as an element that serves to align Thr-44 for pyrimidine binding once Gln-117 has been reoriented. The present findings indicate that in fact neither of these applies. The 44/80 interaction markedly suppresses activity, but by a mechanism that is largely independent of the placement of Gln-117 in the B_1 site. Thus, the changes in transition state binding energy (reflected by k_{cat}/K_m values with small defined substrates) associated with Thr-80 and Gln-117 mutations are mostly or entirely additive (Table 5), and the increase in activity for Q117A/T80A toward tRNA approaches the product of the factors for the two single-residue variants (83- vs 141-fold).

Rather than helping to anchor Gln-117 within the B_1 site, the inhibitory function of Thr-80 is to modulate the recognition of cytosine by Thr-44: the Thr-80 → Ala replacement selectively strengthens activity toward cytidylyl substrates, and this effect is completely eliminated by simultaneous mutation of Thr-44. Thus the 44/80 hydrogen bond is apparently retained in the active conformation of Ang in which substrate is bound, although its length and geometry might be altered. To discern the precise mechanism by which Thr-80 influences Thr-44, it is necessary to first understand the role of the latter residue. In RNase A, the corresponding threonine (Thr-45) forms two hydrogen bonds with both cytosine and uracil; its NH interacts with O2 on each pyrimidine, and its OG can either donate a hydrogen to N3 of cytosine or accept one from N3 of uracil. This versatility may explain the relatively small discrimination by RNase A between the two pyrimidines as proposed by Richards and Wyckoff (*38*) and supported by the similarity of the effects of Thr-45 mutations on activity toward cytidylyl vs uridylyl substrates (*65*). In contrast, Ang shows a 10−20-fold preference for cytidine, and Thr-44 replacement impairs cleavage of CpN dinucleotides much more extensively than that of UpNs (*17*). Moreover, the reductions in activity for Ang are considerably smaller than in RNase A.

A plausible explanation for these findings is that (1) OG of Thr-44 in Ang forms a much more effective hydrogen bond with N3 of cytosine (i.e., as donor) than with that of uracil (where it serves as acceptor) and (2) the additional hydrogen bond of this group with Thr-80 selectively attenuates the interaction with cytosine. When Thr-80 is mutated to Ala, the OG/cytosine hydrogen bond becomes quite strong: replacement of Thr-44 in T80A reduces activity toward CpA by 500-fold, corresponding to a loss of transition-state binding energy of 3.7 kcal/mol. This decrease is comparable to those measured for mutations of the pyrimidine-binding threonines in RNase A (*65*) and another homologue, RNase 4 (*66*), and is much greater than that anticipated for elimination of a single hydrogen bond between uncharged partners (*67*). As suggested by Vicentini et al. (*66*), the unusually extensive activity changes accompanying these substitutions may be due, in part, to adverse effects on other important contacts as well. In any event, the large magnitude and similarity of the consequences of Thr replacement in the two RNases and T80A-Ang suggest that the interaction between the side chains of Thr-

Table 5: Thermodynamic Effects of Various Single- and Multiple-Residue Replacements in Ang

replacement	$\Delta\Delta G^a$ (kcal/mol) with CpA	CpG	C>p
Thr-80 → Ala	1.44	1.59	1.56
Gln-117 → Ala	1.37	1.38	1.48
ARH-I	2.62	1.34	1.91
Thr-80 → Ala/Gln-117 → Ala	2.88 (−0.07)[b]	2.42 (0.55)	2.59 (0.45)
Thr-80 → Ala/ARH-I	3.03 (1.03)	2.23 (0.70)	2.38 (1.09)
Gln-117 → Ala/ARH-I	3.26 (0.73)	2.37 (0.45)	2.56 (0.83)

[a] $\Delta\Delta G$ values represent the changes in free energy of transition state binding for wild-type vs variant Ang and are calculated as $-RT$ ln $[(k_{cat}/K_m)_{wild\text{-}type}/(k_{cat}/K_m)_{variant}]$. Values for UpA are not shown because of the minimal effect of the Thr-80 mutation on activity toward this substrate. [b] Values in parentheses are differences between the measured $\Delta\Delta G$ values for multiple mutant derivatives and the calculated sums of $\Delta\Delta G$ values for the individual mutations. The magnitude of this value indicates the relationship between the effects of the different replacements [ranging from entirely independent (a value of zero) to fully dependent (a value equal to the smaller of the two $\Delta\Delta G$ values for the individual mutations)].

44 and Gln-117 does not contribute significantly to stabilization of the inactive native conformation [although the hydrogen bond between the main chain of Thr-44 and OE1 of Gln-117 (Table 1) might].

The effects of replacing Thr-80 in Ang by Asp, the amino acid at the analogous position (83) in RNase A, reveal further functional similarities and differences between the two enzymes. T80D-Ang, unlike RNase A, exhibits a 2-fold preference for uridylyl vs cytidylyl substrates, resulting from a sizable increase in activity toward the former together with a small decrease in activity toward the latter. Thus, an Asp at position 80 of Ang apparently weakens the hydrogen bond between the Thr-44 OH and cytosine even more extensively than a Thr but strengthens the interaction with N3 of uracil much as Asp-83 does in RNase A (*50*, *51*). An Asp → Ala substitution in RNase A diminishes activity toward uridine nucleotides to nearly the same extent as in T80D-Ang but does not affect reactions with cytidylyl substrates (*51*).

Thr-80 is conserved in mouse, rabbit, and pig Ang, but bovine Ang has an Ile at this position (see ref *61*). Nonetheless, the pyrimidine specificity of the bovine enzyme is similar to that of human Ang and its overall activity is even lower (*68*). In bovine Ang (*59*), there is a water-mediated hydrogen bond between OG of the pyrimidine-binding threonine (Thr-45) and NH1 of Arg-43, another residue unique to the bovine enzyme, which may modulate the pyrimidine interactions of the threonine in the same manner as the 44/80 interaction in human Ang.

Role of Segment 58−*70.* Ang lacks the disulfide loop (65−72 in RNase A) that comprises part of the active center in pancreatic and other mammalian RNases. This loop provides three of the four B_2-site residues seen to form hydrogen bonds with purines in crystal and NMR structures of complexes of nucleotides with RNase A or its proteolytic derivative RNase S: Asn-67, Gln-69, and Asn-71 (*41*, *69*−*71*). The loop also contains Lys-66, which engages in multiple interactions with Asp-121 [66 NZ to 121 O and OD, and 66 N to 121 OD (*53*, *62*)] that may assist the formation of the 119/121 hydrogen bond and/or position B_2-site residues. Lys-66 has been proposed to constitute the P_0 subsite of RNase A as well (*72*). No Ang residues are positioned appropriately to mimic the precise functions of any of the RNase components on this loop, although modeling suggests that Asn-68 may interact with a purine at B_2 (*37*). [Instead, this region constitutes a putative cell-binding site that is essential for angiogenic activity (*47*, *73*, *74*).]

As might be anticipated from these considerations, grafting the RNase A disulfide loop into Ang dramatically enhances ribonucleolytic activity (*34*). However, the present findings suggest that the basis for the increased enzymatic activity of the resultant hybrid, ARH-I, is in fact much more complex. Superposition of the Ang and RNase A structures shows a conflict between the positions of Ser-118 and Lys-66 in the respective proteins (Figure 2). There are two ways in which this might be averted in the hybrid protein: (1) the Lys could adopt the same conformation as in RNase A and Ser-118 is displaced, with concomitant elimination of the 116/118 hydrogen bonds, or (2) the Lys could reorient so as to leave the 116/118 contacts intact. In the first case, the regional replacement would have a substantial impact on the obstructive conformation by removing key stabilizing interactions. In the second case, any contacts of Lys-66 in RNase A would not be replicated in ARH-I. The large magnitude of the activity changes in ARH-I (up to ∼400-fold) favors the first view, and this is further bolstered by the kinetic results obtained here.

ARH-I binds 2′-CMP 10-fold more tightly than does Ang, indicating that the B_1/P_1 region of the hybrid enzyme is improved considerably. It seems likely that a significant part of this change involves the B_1 portion since binding to P_1/B_2 is similarly elevated (ARH-I has 8-fold greater affinity than Ang for 5′-AMP), and the strongly increased selectivity of ARH-I for adenine over guanine (29-fold vs 3-fold for Ang; Table 3) implies that in this case B_2 is affected. Moreover, the ARH-I replacement enhances cleavage of C>p, which lacks a purine moiety, even more than that of CpG (25- vs 10-fold).

The properties of Q117A/ARH-I provide even stronger evidence that the presence of the RNase A segment in ARH-I impinges on the B_1 site. This derivative is only 50% more active toward tRNA than is ARH-I, suggesting either that in ARH-I Gln-117 no longer occupies the pyrimidine site or, if it does, that it contributes little to maintaining the inactive conformation. However, the effects of the Gln-117 and ARH-I replacements on cleavage of dinucleotides and C>p, in terms of free energy changes for transition-state binding calculated from k_{cat}/K_m values, are more additive (Table 5). Thus the orientation of the C-terminal segment in ARH-I may still be obstructive (but less stable) or, alternatively, the Gln-117 side chain in ARH-I might attenuate activity toward small substrates by some separate mechanism. It should be noted that mutation of Gln-117 in D116A also increases effectiveness with some substrates (*36*).

Surprisingly, the creation of ARH-I also neutralizes much of the negative role of Thr-80: T80A/ARH-I has only marginally increased activity toward tRNA compared to ARH-I, and the effects of the T80A and ARH-I mutations with CpN dinucleotides and C>p are even less additive than for Q117A/ARH-I (Table 5). At this point, there is no satisfactory structural explanation for the influence of the RNase A residues 59−73 on Thr-80, particularly in light of the considerable independence of the consequences of the T80A and Q117A mutations. If the RNase segment eliminates the 44/80 hydrogen bond without affecting Thr-44, this would be expected to produce a major increase in specificity for CpN substrates. However, the C vs U preference of T80A/ARH-I is actually somewhat lower than that of Ang; thus, the ARH-I replacement may alter the manner in which Thr-44 interacts with pyrimidines such that the modulatory role of Thr-80 is largely eliminated.

The double mutant Q117A/T80A is even more active toward cytidylyl substrates than is ARH-I, and its activity with CpG and C>p is indistinguishable from that of Q117A/ARH-I. Given the substantial degree of interdependence between the effects of the ARH-I mutation and those accompanying the Thr-80 and Gln-117 replacements, it can be inferred that the majority of the activity increase for ARH-I vs Ang derives from factors other than improvement of the B_2 site. Thus, the significance of the differences between Ang and RNase A in this region is much broader than was initially apparent. It is possible that the existence of the 65−72 disulfide loop is an important contributing

factor in preventing the adoption of an Ang-like C-terminal conformation in those mammalian RNases that, like RNase A, contain the other elements that can potentially participate in stabilization. In this regard, two reverse hybrids of ARH-I (in which Ang segments 62−71 and 58−70 have been introduced into RNase A) have 60- and 23-fold lower activity, respectively, than does RNase A (*75*, *76*). These changes may reflect, in part, the formation of Asp-121/Ser-123 interactions such as those between Asp-116 and Ser-118 in Ang.

Other Features of Ang That Attenuate Enzymatic Activity. The three factors discussed thus far do not account for the entire difference in enzymatic potency between Ang and RNase A. The most active of the variants generated here (Q117A/ARH-I) remains 100-fold (with tRNA) to >1000-fold (with dinucleotides) less active than RNase A, and on the basis of our present understanding, it seems unlikely that simultaneous substitutions of other residues connected with these factors would lead to any further large increase in activity. Two additional features of Ang have been demonstrated or proposed to contribute to its catalytic weakness. Replacement of the Ang segment 38−41 by its counterpart in RNase A (38−42), producing the hybrid protein ARH-II, enhances ribonucleolytic activity by 5−75-fold (*35*). The structural basis for this increase has not been established. However, the properties of a derivative incorporating both the ARH-I and ARH-II substitutions indicate that the activity changes produced in the two cases are not independent. Perhaps the ARH-II mutation influences Thr-44 and Thr-80 in much the same manner as for ARH-I. The presence of Leu at position 115 in Ang, where all pancreatic RNases have Phe or Tyr, has also been suggested to attenuate activity since Phe → Leu substitution in a semisynthetic RNase A derivative decreases potency by 7-fold (*77*), and the reverse replacement in an RNase A (1−118)−Ang (108−123) noncovalent hybrid protein improves activity by as much as 100-fold (*78*). However, it should be noted that bovine and mouse Ang both have Phe at this position and yet are even less active than the human protein (*68*, *79*).

Some of the structural characteristics of Ang that underlie its low enzymatic activity remain to be elucidated. It is possible that multiple additional considerations come into play in minor ways. The determination of three-dimensional structures of Ang−nucleotide complexes, which has not yet proved feasible, should ultimately shed light on this problem and provide a more complete understanding of the detailed mechanisms by which the factors already identified attenuate activity.

Biological Significance of the Enzymatic Weakness of Ang. Why, from a physiological point of view, is the ribonucleolytic activity of Ang toward standard substrates so low? Although this question cannot be answered definitively until the in vivo substrate of Ang has been identified, some speculation seems warranted. Evidence to date suggests that Ang may exert its enzymatic activity in the nucleolus of target cells: Ang binds to cell-surface receptors on endothelial cells (*24*) and is subsequently translocated to the nucleolus (*80*). This translocation, which is an essential step in the induction of angiogenesis, brings Ang into direct contact with numerous potential RNA substrates. If the nucleolus is indeed where Ang acts (e.g., participating in the production of the new ribosomes required for cell proliferation), the apparent weakness of its activity is readily understandable since a potent general RNase would no doubt wreak havoc in this location.

As discussed above, the structural basis for Ang's attenuated activity is extremely elaborate and involves at least four or five independent factors. Thus it is clear that the "purpose" served is not merely the loss of overall enzymatic potency, which could have been achieved more simply; e.g., mutation of either catalytic histidine of RNase A reduces activity by a similar factor (*81*). Ang may have evolved essentially to be a latent enzyme that undergoes activation at the appropriate time and place in vivo. Such activation might be accomplished, in principle, either by binding an effector molecule or by proteolytic processing. However, the latter now seems unlikely in view of the large number of discontinuous regions that participate in the suppression of activity and the positive role of the C-terminal residues 121−123 (*44*). Even without being activated, Ang may have a much more robust activity toward some specific RNA substrate, e.g. in the nucleolus. It is also possible, of course, that the activity of Ang, although small, is nevertheless sufficient to accomplish the required task, as appears to be the case for other weak enzymes such as CMV protease (*82*).

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REFERENCES

1. D'Alessio, G., and Riordan, J. F. (1997) *Ribonucleases. Structures and Functions.* Academic Press, New York.
2. Fett, J. W., Strydom, D. J., Lobb, R. R., Alderman, E. M., Bethune, J. L., Riordan, J. F., and Vallee, B. L. (1985) *Biochemistry 24*, 5480−5486.
3. King, T. V., and Vallee, B. L. (1991) *J. Bone Joint Surg. 73-B*, 587−590.
4. Olson, K. A., French, T. C., Vallee, B. L., and Fett, J. W. (1994) *Cancer Res. 54*, 4576−4579.
5. Olson, K. A., Fett, J. W., French, T. C., Key, M. E., and Vallee, B. L. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 442−446.
6. Matousek, J. (1973) *Experientia 29*, 858−859.
7. Laccetti, P., Spalletti-Cernia, D., Portella, G., De Corato, P., D'Alessio, G., and Vecchio, G. (1994) *Cancer Res. 54*, 4253−4256.
8. Mikulski, S. M., Ardelt, W., Shogen, K., Bernstein, E. H., and Menduke, H. (1990) *J. Natl. Cancer Inst. 82*, 151−153.
9. Soucek, J., Chudomel, V., Potmesilova, I., and Novak, J. T. (1986) *Nat. Immun. Cell Growth Regul. 5*, 250−258.
10. Gleich, G. J., Loegering, D. A., Bell, M. P., Checkel, J. L., Ackerman, S. J., and McKean, D. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3146−3150.
11. Hamann, K. J., Barker, R. L., Loegering, D. A., and Gleich, G. J. (1987) *J. Parasitol. 73*, 523−529.
12. Ackerman, S. J., Gleich, G. J., Loegering, D. A., Richardson, B. A., and Butterworth, A. E. (1985) *Am. J. Trop. Med. Hyg. 34*, 735−745.
13. Lehrer, R. I., Szklarek, D., Barton, A., Ganz, T., Hamann, K. J., and Gleich, G. J. (1989) *J. Immunol. 142*, 4428−4434.
14. Rosenberg, H. F. (1995) *J. Biol. Chem. 270*, 7876−7881.

15. Shapiro, R., Fox, E. A., and Riordan, J. F. (1989) *Biochemistry 28*, 1726−1732.
16. Shapiro, R., and Vallee, B. L. (1989) *Biochemistry 28*, 7401−7408.
17. Curran, T. P., Shapiro, R., and Riordan, J. F. (1993) *Biochemistry 32*, 2307−2313.
18. Vescia, S., Tramontano, D., Augusti-Tocco, G., and D'Alessio, G. (1980) *Cancer Res. 40*, 3740−3744.
19. Kim, J. S., Soucek, J., Matousek, J., and Raines, R. T. (1995) *Biochem. J. 308*, 547−550.
20. Sorrentino, S., Glitz, D. G., Hamann, K. J., Loegering, D. A., Checkel, J. L., and Gleich, G. J. (1992). *J. Biol. Chem. 267*, 14859−14865.
21. Newton, D. L., Walbridge, S., Mikulski, S. M., Ardelt, W., Shogen, K., Ackerman, S. J., Rybak, S. M., and Youle, R. J. (1994) *J. Neurosci. 14*, 538−544.
22. Kim, J. S., Soucek, J., Matousek, J., and Raines, R. T. (1995) *J. Biol. Chem. 270*, 31097−31102.
23. Hu, G.-f., Riordan, J. F., and Vallee, B. L. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12096−12100.
24. Hu, G.-f., Riordan, J. F., and Vallee, B. L. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 2204−2209.
25. Shapiro, R., Riordan, J. F., and Vallee, B. L. (1986) *Biochemistry 25*, 3527−3532.
26. Irie, M., and Hosokawa, S. (1971) *J. Biochem. (Tokyo) 70*, 301−309.
27. Floridi, A., D'Alessio, G., and Leone, E. (1972) *Eur. J. Biochem. 26*, 162−167.
28. Slifman, N. R., Loegering, D. A., McKean, D. J., and Gleich, G. J. (1986) *J. Immunol. 137*, 2913−2917.
29. Sorrentino, S., Tucker, G. K., and Glitz, D. G. (1988) *J. Biol. Chem. 263*, 16125−16131.
30. Shapiro, R., and Vallee, B. L. (1991) *Biochemistry 30*, 2246−2255.
31. Sorrentino, S., and Glitz, D. G. (1991) *FEBS Lett. 288*, 23−26.
32. Boix, E., Wu, Y., Vasandani, V. M., Saxena, S. K., Ardelt, W., Ladner, J., and Youle, R. J. (1996) *J. Mol. Biol. 257*, 992−1007.
33. Shapiro, R., Harper, J. W., Fox, E. A., Jansen, H.-W., Hein, F., and Uhlmann, E. (1988) *Anal. Biochem. 175*, 450−461.
34. Harper, J. W., and Vallee, B. L. (1989) *Biochemistry 28*, 1875−1884.
35. Harper, J. W., Fox, E. A., Shapiro, R., and Vallee, B. L. (1990) *Biochemistry 29*, 7297−7302.
36. Russo, N., Shapiro, R., Acharya, K. R., Riordan, J. F., and Vallee, B. L. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 2920−2924.
37. Russo, N., Acharya K. R., Vallee, B. L., and Shapiro, R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 804−808.
38. Richards, F. M., and Wyckoff, H. W. (1971) *Enzymes (3rd Ed.) 4*, 647−806.
39. Nogues, M. V., Vilanova, M., and Cuchillo, C. M. (1995) *Biochim. Biophys. Acta 1253*, 16−24.
40. Acharya, K. R., Shapiro, R., Allen, S. C., Riordan, J. F., and Vallee, B. L. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 2915−2919.
41. Richards, F. M., and Wyckoff, H. W. (1973) in *Atlas of Molecular Structures in Biology-1: Ribonuclease S* (Phillips, D. C., and Richards, F. M., Eds.) Oxford University Press, London.
42. Borah, B., Chen, C., Egan, W., Miller, M., Wlodawer, A., and Cohen, J. S. (1985) *Biochemistry 24*, 2058−2067.
43. Harper, J. W., and Vallee, B. L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7139−7143.
44. Russo, N., Nobile, V., Di Donato, A., Riordan, J. F., and Vallee, B. L. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 3243−3247.
45. Shapiro, R., and Vallee, B. L. (1992) *Biochemistry 31*, 12477−12485.
46. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene 77*, 51−59.
47. Hallahan, T. W., Shapiro, R., Strydom, D. J., and Vallee, B. L. (1992) *Biochemistry 31*, 8022−8029.
48. Shapiro, R., Weremowicz, S., Riordan, J. F., and Vallee, B. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8783−8787.
49. Shapiro, R., Fett, J. W., Strydom, D. J., and Vallee, B. L. (1986) *Biochemistry 25*, 7255−7264.
50. Wlodawer, A., Miller, M., and Sjolin, L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3628−3631.
51. delCardayre, S. B., and Raines, R. T. (1995) *J. Mol. Biol. 252*, 328−336.
52. Curran, T. P., Shapiro, R., Riordan, J. F., and Vallee, B. L. (1993) *Biochim. Biophys. Acta 1202*, 281−286.
53. Wlodawer, A., Svensson, L. A., Sjolin, L., and Gilliland, G. L. (1988) *Biochemistry 27*, 2705−2717.
54. Howlin, B., Moss, D. S., and Harris, G. W. (1989) *Acta Crystallogr. A48*, 851−861.
55. Leonidas, D. D., Shapiro, R., Irons, L. I., Russo, N., and Acharya, K. R. (1997) *Biochemistry 36*, 5578−5588.
56. Mosimann, S. C., Newton, D. L., Youle, R. J., and James, M. N. G. (1996) *J. Mol. Biol. 260*, 540−552.
57. Mazzarella, L., Capasso, S., Demasi, D., Di Lorenzo, G., Mattia, C. A., and Zagari, A. (1993) *Acta Crystallogr. D49*, 389−402.
58. Mosimann, S. C., Ardelt, W., and James, M. N. G. (1994) *J. Mol. Biol. 236*, 1141−1153.
59. Acharya, K. R., Shapiro, R., Riordan, J. F., and Vallee, B. L. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2949−2953.
60. Lequin, O., Albaret C., Bontems, F., Spik, G., and Lallemand, J.-Y. (1996) *Biochemistry 35*, 8870−8880.
61. Bond, M. D., Strydom, D. J., and Vallee, B. L. (1993) *Biochim. Biophys. Acta 1162*, 177−186.
62. deMel, V. S. J., Martin, P. D., Doscher, M. S., and Edwards, B. F. P. (1992) *J. Biol. Chem. 267*, 247−256.
63. Hodges, R. S., and Merrifield, R. B. (1975) *J. Biol. Chem. 250*, 1231−1241.
64. Gilliland, G. L., Dill, J., Pechik, I., Svensson, L. A., and Sjolin, L. (1994) *Protein Pept. Lett. 1*, 60−65.
65. delCardayre, S. B., and Raines, R. T. (1994) *Biochemistry 33*, 6031−6037.
66. Vicentini, A. M., Kote-Jarai, Z., and Hofsteenge, J. (1996) *Biochemistry 35*, 9128−9132.
67. Fersht, A. R., Shi, J.-P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y., and Winter, G. (1985) *Nature 314*, 235−238.
68. Strydom, D. J., Bond, M. D., and Vallee, B. L. (1997) *Eur. J. Biochem. 247*, 535−544.
69. Wodak, S. Y., Liu, M. Y., and Wyckoff, H. W. (1977) *J. Mol. Biol. 116*, 855−875.
70. Zegers, I., Maes, D., Dao-Thi, M.-H., Poortmans, F., Palmer, R., and Wyns, L. (1994) *Protein Sci. 3*, 2322−2339.
71. Toiron, C., Gonzalez, C., Bruix, M., and Rico, M. (1996) *Protein Sci. 5*, 1633−1647.
72. Mitsui, Y., Urata, Y., Torii, K., and Irie, M. (1978) *Biochim. Biophys. Acta 535*, 299−308.
73. Hallahan, T. W., Shapiro, R., and Vallee, B. L. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2222−2226.
74. Hu, G.-f., Strydom, D. J., Fett, J. W., Riordan, J. F., and Vallee, B. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1217−1221.
75. Allemann, R. K., Presnell, S. R., and Benner, S. A. (1991) *Protein Eng. 4*, 831−835.
76. Raines, R. T., Toscano, M. P., Nierengarten, D. M., Ha, J. H., and Auerbach, R. (1995) *J. Biol. Chem. 270*, 17180−17184.
77. Lin, M. C., Gutte, B., Caldi, D. G., Moore, S., and Merrifield, R. B. (1970) *J. Biol. Chem. 247*, 4768−4774.
78. Harper, J. W., Auld, D. S., Riordan, J. F., and Vallee, B. L. (1988) *Biochemistry 27*, 219−226.
79. Nobile, V., Vallee, B. L., and Shapiro, R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 4331−4335.
80. Moroianu, J., and Riordan, J. F. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1677−1681.
81. Thompson, J. E., and Raines, R. T. (1994) *J. Am. Chem. Soc. 116*, 5467−5468.
82. Babè, L. M., and Craik, C. S. (1997) *Cell 91*, 427−430.
83. Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946−950.

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