Alteration of the Enzymatic Specificity of Human Angiogenin by Site-Directed Mutagenesis†

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Received November 25, 1992; Revised Manuscript Received December 29, 1992

ABSTRACT: The molecular basis for the enzymatic specificity of human angiogenin has been investigated by site-directed mutagenesis of Thr-44, Glu-108, and Ser-118—residues corresponding to those thought to be involved in substrate base recognition in the homologous protein, RNase A. Mutations of Thr-44 to Ala, His, and Asp affect both activity and specificity dramatically. The Ala and His replacements decrease activity toward tRNA by factors of 25 and 40, respectively, and reduce cleavage of cytidylyl more than uridylyl dinucleotides. Substitution by Asp does not influence the rate of tRNA and rRNA degradation but alters specificity even more markedly than the other mutations: T44D-angiogenin has 17–40-fold decreased activity toward CpN′ dinucleotides and 1.3–1.9-fold increased activity toward UpN′, resulting in an inverted order of preference (U > C) compared to native angiogenin. Mutations of Glu-108 to Lys and Gln change activity toward RNA and dinucleotides by no more than 50% and produce slight increases in preference for adenosine vs guanosine at position N′ of NpN′ substrates. Mutations of Ser-118 to Asp and Arg have a larger effect, decreasing activity by factors of ~2 and 4, respectively, toward all substrates examined. These results indicate that (i) Thr-44 is important for recognition of the pyrimidine moiety at position N, (ii) Glu-108 may make a small contribution to binding the N′-nucleotide, and (iii) Ser-118 has a minor functional role, which appears to involve catalysis rather than nucleotide binding. The angiogenic activities of T44D- and T44H-angiogenin on the chick embryo chorioallantoic membrane correlate with their enzymatic activities toward RNA: T44D is fully active, whereas T44H has vastly decreased potency. This suggests that Thr-44 plays a critical role in angiogenesis that derives from its contribution to ribonucleolytic activity.

Human angiogenin, a 14.1-kDa monomeric protein (Fett et al., 1985), is an unusual member of the pancreatic ribonuclease superfamily (Strydom et al., 1985; Kurachi et al., 1985): it induces neovascularization in vivo (Fett et al., 1985; Denèfle et al., 1987; King & Vallee, 1991) and exerts a variety of effects on vascular endothelial and smooth muscle cells in vitro (Bicknell & Vallee, 1988,1989; Moore & Riordan, 1990; Soncin, 1992). The ribonucleolytic activity of angiogenin, which differs markedly from that of pancreatic RNase[1] A in both magnitude and specificity (Shapiro et al., 1986; Rybak & Vallee, 1988), is necessary but not sufficient for angiogenic activity. Thus, both activities are abolished by site-directed mutagenesis of His-13, His-114, or Lys-40 in the active site (Shapiro & Vallee, 1989; Shapiro et al., 1989), whereas changes in another region, the putative cell-binding site, only diminish angiogenic but not enzymatic activity (Hallahan et al., 1991, 1992).

Since cleavage of RNA or a related substrate appears to comprise part of the molecular mechanism by which angiogenin induces blood vessel formation, it is of considerable interest to determine the structural basis for this enzymatic specificity. X-ray crystallographic studies of bovine pancreatic RNase A (Richards & Wyckoff, 1973; Wodak et al., 1977; Borkakoti et al., 1982; Borkakoti, 1983; Wlodawer, 1985), whose primary sequence is 33% identical to that of angiogenin (Strydom et al., 1985; Kurachi et al., 1985), have implicated six residues in substrate base recognition, with Thr-45 and Ser-123 forming the so-called B_1 site where the 3′-nucleotide binds and Asn-67, Gln-69, Asn-71, and Glu-111 comprising the B_2 site for the 5′-nucleotide. Both residues in the B_1 site and one of those in the B_2 site (Glu-111) are conserved in angiogenin. The remaining three amino acids are part of an exposed loop in RNase that differs greatly from its angiogenin counterpart in both primary and secondary structure. Thus, neither Asn-67 nor Gln-69 is conserved, and although the usual angiogenin/RNase sequence alignment (Strydom et al., 1985) shows Asn-68 of angiogenin as corresponding to Asn-71 of RNase, its position with respect to the catalytic histidines and lysine in the calculated three-dimensional structure of angiogenin (Palmer et al., 1986) is dissimilar.

The present mutagenesis study has investigated the functional roles of Thr-44, Glu-108, and Ser-118, the angiogenin residues corresponding to those proposed to be in the B_1 and B_2 sites of RNase. Replacements of these amino acids influence ribonucleolytic activity to widely varying extents. The Glu-108 mutations produce small, substrate-dependent changes, indicating perhaps a minor contribution by this residue to substrate specificity. The consequences of the Ser-118 substitutions are more pronounced: they decrease activity toward all substrates examined by a factor of 2–4 and in this case suggest a somewhat greater functional role, which may

† This work was supported by funds from Hoechst, A.G., under agreements with Harvard University. T.P.C. was supported by National Research Service Award HL-07823-02 from the National Heart, Lung and Blood Institute.

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[1] Abbreviations: RNase, ribonuclease; RNase A, bovine pancreatic ribonuclease A; CAM, chorioallantoic membrane; HPLC, high-performance liquid chromatography; C18, octadecylsilane; NpN′, dinucleoside 3′,5′-phosphate; RNase S, ribonuclease A that has been proteolytically cleaved between residues 19 and 20, 20 and 21, or 21 and 22; U>p, uridine cyclic 2′,3′-phosphate; C>p, cytidine cyclic 2′,3′-phosphate.

0006-2960/93/0432-2307$04.00/0

not, however, involve nucleotide binding. Mutations of Thr-44 have the most dramatic effect on activity and specificity, which points to a major role for this residue in substrate recognition. The importance of Thr-44 for angiogenic activity has also been examined.

EXPERIMENTAL PROCEDURES

Site-directed mutagenesis was performed by the method of Kunkel (1985) as described previously (Shapiro et al., 1988). The mutagenic oligonucleotides were pGATATCAAC GATTTCATCCAT (T44D), pGATATCAACGCTTTC-ATCCAT (T44A), pGATATCAACCATTTCATCCAT (T44H), pGTTGCTTGTAAAAACGGTCTG (E108K), pGTTGCTTGTCAAAACGGTCTG (E108Q), pCTA-GATCAGGATATCTTCCGA (S118D), and pCTAGAT-CAGCGTATCTTCCGA (S118R). DNA sequencing of M13 clones identified the desired mutants and ruled out the possibility of spurious mutations.

Protein concentrations were measured by amino acid analysis (Strydom et al., 1985). Tryptic peptide mapping was performed as described previously (Hallahan et al., 1992), as were enzymatic assays with tRNA (Shapiro et al., 1987), 18S and 28S rRNA (Shapiro et al., 1986), and dinucleotides (Shapiro et al., 1986; Harper & Vallee, 1989). Angiogenic activity was assessed on the chick embryo chorioallantoic membrane (CAM) (Knighton et al., 1977; Fett et al., 1985). The numbers of positive and negative responses for each sample from multiple sets of assays were combined and χ^2 values were calculated from an outcome contingency table by comparing the test sample with a water control; the associated probabilities, p, were then obtained (Hallahan et al., 1992). A value of $p < 0.05$ is needed for a sample to be considered active (Fett et al., 1985).

RESULTS

Preparation of Angiogenin Mutants. Mutant proteins were expressed in *Escherichia coli* strain W3110 as Met-(−1)[2] derivatives and purified to homogeneity from inclusion bodies by established procedures (Shapiro et al., 1988). Yields ranged from 0.12 mg/L (E108Q) to 1.8 mg/L (S118D). The retention time of each mutant protein during Mono S HPLC is consistent with the amino acid replacement: T44D and S118D elute slightly earlier than angiogenin, E108K, E108Q, and S118R somewhat later, and T44A and T44H at the same time as angiogenin. Final preparations were >98% pure as judged by SDS–PAGE.

Structural Characterization. The amino acid compositions of all mutant proteins are consistent with those expected (data not shown). Substitution of Gln for Glu in E108Q can be inferred from its chromatographic properties (see above). Tryptic digest C18 HPLC elution profiles are identical to that of angiogenin except for peptides containing the substituted amino acids [angiogenin tryptic peptides are designated according to Strydom et al. (1985)] and demonstrate that the three disulfide bonds have formed properly in all instances. The amino acid compositions of peptide T8 (residues 41–50) for T44D, T44A, and T44H confirm the Asp, Ala, or His replacements, respectively, and rule out other changes (Table I). In native angiogenin, residues 108 and 118 are both components of peptide T11 (amino acids 55–60 linked by disulfide bond to amino acids 102–121). T11 of E108Q is identical to the angiogenin peptide in amino acid composition but elutes earlier, consistent with the Glu → Gln substitution. The amino acid composition of T11 of S118D reveals the Ser → Asp substitution (Table II). In both E108K and S118R, the mutation introduces a new tryptic cleavage site. Therefore, peptide T11 is absent from the maps of these derivatives, and in each case two additional peptides are present. The amino acid compositions of the E108K and S118R peptides containing the replacements are in accord with the proposed structures (Table II).

Ribonucleolytic Activity. The activities of the mutant proteins toward tRNA vary widely (Table III). T44D is almost as potent as the native enzyme, whereas T44A and T44H have markedly reduced activities, 4% and 2% of angiogenin's, respectively. The activities of the Glu-108 mutants are only increased (E108Q) or decreased (E108K) slightly. Replacements of Ser-118 diminish activity by 2.5-fold (S118R) and 4-fold (S118D).

Native angiogenin catalyzes the limited cleavage of 28S and 18S rRNA, yielding fragments 100–500 nucleotides in length that are relatively resistant to further degradation (Shapiro et al., 1986). All seven mutant proteins generate

Table I: Amino Acid Compositions of Tryptic Peptide T8 (Amino Acids 41–50) from Thr-44 Mutants of Angiogenin

	mutants[a]		
amino acid	T44D	T44A[b]	T44H
Asx	3.96 (4)	2.64 (3)	2.69 (3)
Gly	0.92 (1)	1.33 (1)	1.12 (1)
His	0.89 (1)	1.43 (1)	1.78 (2)
Thr			
Ala		1.06 (1)	
Ile	1.23 (2)	1.69 (2)	1.68 (2)
Phe	0.80 (1)	0.90 (1)	0.93 (1)
Lys	0.89 (1)	0.89 (1)	0.88 (1)
pmol analyzed	128	87	236

[a] Numbers in parentheses represent expected compositions from DNA sequence of each mutant protein. Amino acids present in quantities less than 0.1 residue are not indicated. Cystine and tryptophan contents were not determined. [b] The amino acid composition of the T44A peptide has been corrected for the presence of 0.33 molar equiv of peptide T9.

Table II: Amino Acid Compositions of Selected Tryptic Peptides from E108K, S118D, and S118R[a]

	mutants		
amino acid	E108K	S118D	S118R[b]
Asx	1.63 (2)	5.63 (5)	4.07 (4)
Glx	0.99 (1)	2.92 (3)	3.10 (3)
Ser			
Gly		1.26 (1)	1.18 (1)
His		1.01 (1)	1.10 (1)
Arg		1.03 (1)	1.05 (1)
Ala	2.11 (2)	1.95 (2)	2.18 (2)
Pro		1.15 (1)	1.18 (1)
Val	1.99 (3)	2.98 (4)	3.09 (4)
Ile	0.78 (1)	1.80 (2)	0.97 (1)
Leu		2.02 (2)	1.99 (2)
Phe		0.95 (1)	
Lys	1.65 (2)	1.06 (1)	1.09 (1)
pmol analyzed	183	150	161

[a] Each peptide shown corresponds to a new peak in the tryptic map of the mutant protein indicated. Numbers in parentheses represent compositions expected for E108K, S118D, and S118R peptides containing amino acids 55–60 disulfide-linked to amino acids 102–108, 102–121, and 102–118, respectively. Amino acids present in quantities less than 0.1 residue are not indicated. Cystine and tryptophan contents were not determined. [b] The amino acid composition of the S118R peptide has been corrected for the presence of 0.31 molar equiv of peptide T10.

[2] Met-(−1) angiogenin is indistinguishable from the native <Glu-1 protein with respect to angiogenic and ribonucleolytic activities (Shapiro et al., 1988).

Table III: Relative Activities of Angiogenin and Angiogenin Mutants toward tRNA[a]

enzyme	rel activity	enzyme	rel activity
angiogenin	1.00	E108K	0.67
T44D	0.85	E108Q	1.40
T44A	0.04	S118D	0.25
T44H	0.02	S118R	0.40

[a] Activities were determined in 33 mM Hepes and 33 mM NaCl, pH 6.8, at 37 °C as described (Shapiro et al., 1987).

Table IV: Enzymatic Activity of Angiogenin and Angiogenin Mutants toward Dinucleotides[a]

	k_{cat}/K_m (M^{-1} s^{-1})			
enzyme	CpA	UpA	CpG	UpG
angiogenin	12	1.1	4.0	0.4
T44A	0.5	0.4	0.1	0.07
T44H	2.3	1.1	0.2	0.1
T44D	0.7	2.1	0.1	0.5
E108K	14	1.5	3.5	0.3
E108Q	16	1.6	2.4	0.2
S118D	7.3	0.6	2.1	0.2
S118R	3.2	0.6	0.7	0.1

[a] Activities were measured in 33 mM Mes and 33 mM NaCl, pH 5.9, at 37 °C. Values for angiogenin are from Harper and Vallee (1989).

this same pattern, as judged by agarose gel electrophoresis (data not shown). The specific activities of T44D, E108K, E108Q, S118D, and S118R in this assay are indistinguishable from that of angiogenin, whereas T44A and T44H are about 12- and 25-fold less active, respectively.

In order to examine the base specificity of the mutant enzymes, k_{cat}/K_m values for cleavage of NpN′ dinucleotides were determined (Table IV). Native angiogenin has a 10-fold preference for cytidine vs uridine at position N and a 3-fold preference for adenosine vs guanosine at position N′; the resultant order of reactivity is CpA > CpG > UpA > UpG. All of the Thr-44 mutants have altered specificities. This change is most dramatic for T44D: activity toward UpA and UpG is increased by 1.9- and 1.3-fold, respectively, while that toward CpA and CpG is decreased by 17- and 40-fold, respectively, resulting in an overall 30–50-fold change in selectivity and an inverted order of preference (U > C) at position N. With T44A and T44H the order remains C > U, but activity toward CpN′ decreases much more extensively than toward UpN′ substrates, and both derivatives therefore have greater activity toward UpA than CpG. In addition to these changes in base recognition at position N, all three mutants, but particularly T44H, exhibit an increased preference for adenosine over guanosine at position N′.

Mutation of Glu-108 to Gln or Lys produces only minor changes in base specificity. Both E108K and E108Q have slightly increased activity (by 15–45%) vs NpA and decreased activity (by 12–50%) vs NpG substrates. The specificities of S118D and S118R are essentially the same as that of angiogenin, and activities toward all four dinucleotides are decreased to an extent that is similar to that measured with tRNA as substrate.

Angiogenic Activity. The angiogenic activities of the mutant derivatives showing the greatest changes in enzymatic activity toward tRNA (T44H) and substrate specificity with dinucleotides (T44D) were assessed on the CAM (Table V). T44H has substantially diminished potency: at 10 ng/egg the percent positive response is much lower than that for angiogenin at 0.1 ng/egg and is statistically indistinguishable from that obtained with water control samples. In contrast, T44D retains activity comparable to that of angiogenin.

Table V: Angiogenic Activity of Angiogenin, T44D, and T44H[a]

sample	dose (ng)	% positives (n)[b]	p[c]
angiogenin	10	52 (78)	<0.0001
	1	40 (35)	0.0006
	0.1	50 (20)	0.003
T44D	10	49 (43)	0.0002
	1	47 (72)	<0.0001
	0.1	38 (71)	0.005
T44H	10	16 (49)	0.66

[a] The CAM assay was employed (Knighton et al., 1977; Fett et al., 1985; Hallahan et al., 1992). Each mutant protein sample was tested in at least three separate experiments utilizing 8–20 eggs. [b] The total number of assays performed for each sample, n, is given in parentheses. [c] Significance, p, was calculated from χ^2 values, based on comparison with water control samples, which produced a 19% positive response (total of 109 eggs).

DISCUSSION

Angiogenin is several orders of magnitude less active than RNase A toward the substrates commonly employed for pancreatic RNase (Shapiro et al., 1986, 1988; Harper & Vallee, 1989), and exhibits a distinct, characteristic specificity toward dinucleotides (Harper & Vallee, 1989), 18S and 28S rRNA (Shapiro et al., 1986; St. Clair et al., 1987) and tRNA (Rybak & Vallee, 1988). Nonetheless, it shares with RNase A certain general features of base specificity, most notably an almost absolute requirement for a 3′-pyrimidine, preferably cytosine, and a 5′-nucleotide selectivity A > G > C > U (Rybak & Vallee, 1988; Shapiro et al., 1988; Harper & Vallee, 1989; Witzel & Barnard, 1962; R.S., unpublished results). In view of the strong overall sequence homology between RNase and angiogenin, it seems likely that these functional similarities reflect structural elements common to both proteins. In the present work, we have therefore explored the molecular basis for the specificity of angiogenin, which is critical for its biological activity, by mutating the residues in angiogenin corresponding to those proposed to be responsible for substrate recognition in RNase A.

The combined results of numerous studies of pancreatic RNase employing X-ray and neutron diffraction (Richards & Wyckoff, 1973; Wodak et al., 1977; Borkakoti et al., 1982; Borkakoti, 1983; Wlodawer, 1985), molecular dynamics (Brünger et al., 1985), and amino acid substitution (Hodges & Merrifield, 1975; Miranda, 1990) suggest that two residues—Thr-45 and Ser-123—are involved in the B_1, i.e., pyrimidine, binding site and that as many as four—Asn-67, Gln-69, Asn-71, and Glu-111—may interact with the base in the B_2 site optimally suited for adenine. Thr-45 is thought to participate through both its peptide NH and its β-hydroxyl group (Figure 1A,B). Thus, in the crystal structures of inhibitor complexes with RNase A and RNase S as well as in molecular dynamics simulations, the peptide NH hydrogen bonds to O(2) of both uracil and cytosine, and the β-OH group can either accept a hydrogen from N(3) of uracil or donate one to N(3) of cytosine. Evidence for the side-chain interactions has also been obtained by site-directed mutagenesis (Miranda, 1990; see below).

In contrast with Thr-45, Ser-123 of RNase appears to interact only with uracil. Crystallographic studies indicate that its β-OH can donate a hydrogen to the carbonyl oxygen at position 4 of uracil (Richards & Wyckoff, 1973; Borkakoti et al., 1982) but that the corresponding bond involving cytosine—i.e., with the serine OH accepting a hydrogen from the amino group at position 4—is not formed (Wodak et al.,

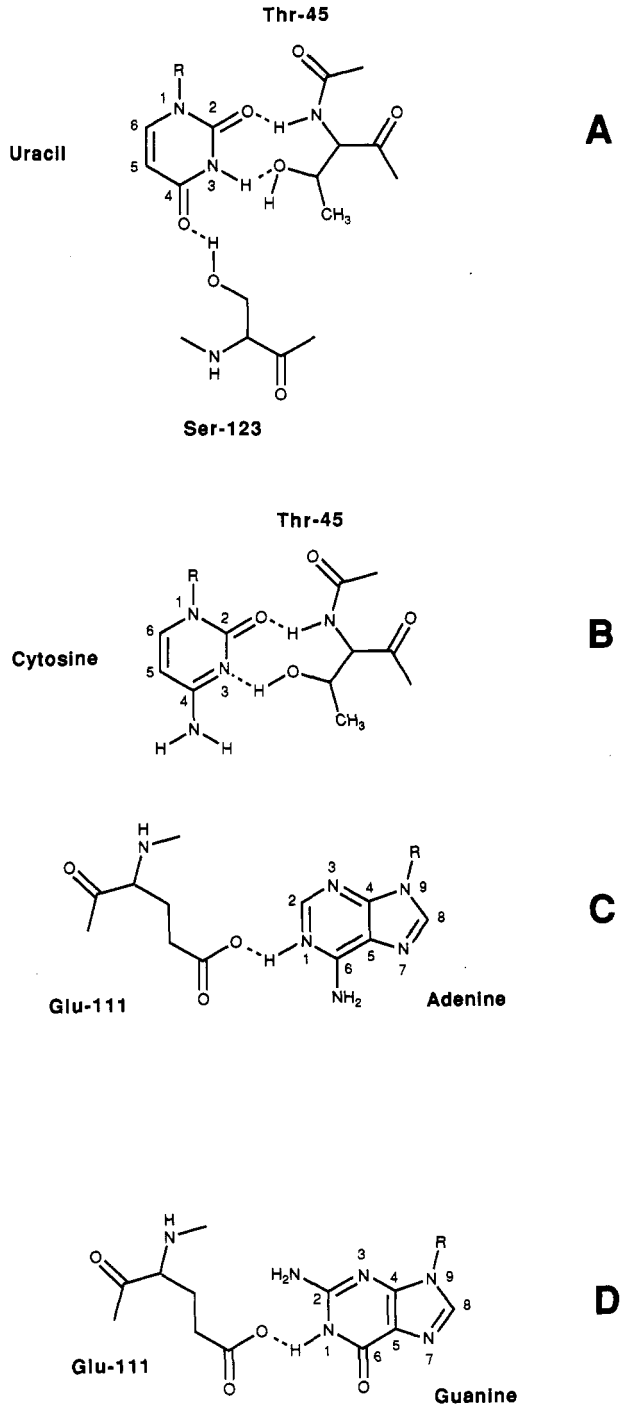

FIGURE 1: Proposed substrate binding roles for Thr-45, Glu-111, and Ser-123 in RNase A (Richards & Wyckoff, 1973; Wodak et al., 1977; Borkakoti et al., 1982; Borkakoti, 1983; Wlodawer, 1985). Dashed lines indicate hydrogen bonds. (A) Interaction of Thr-45 and Ser-123 with uracil; (B) interaction of Thr-45 with cytosine; (C) interaction of Glu-111 with adenine; (D) interaction of Glu-111 with guanine.

1977; Borkakoti et al., 1982). This view is supported by the finding that a semisynthetic [Ala-123] derivative of RNase has decreased activity toward U>p and poly(uridylic acid) but unchanged activity toward C>p and poly(cytidylic acid) (Hodges & Merrifield, 1975). Even for uracil-containing substrates, however, the contribution of this residue is not substantial since the activity loss is only 2–4-fold. The small (55%) decrease in activity toward RNA following enzymatic removal of residues 123 and 124 (Potts et al., 1964) also points to a supplementary rather than a crucial role for Ser-123.

The B_2 site of RNase A has not been examined in as much detail as B_1, and it remains unclear precisely which side chains participate. In the X-ray structure of the RNase S complex with cytidylyl-2′,5′-adenosine (Wodak et al., 1977), one of the γ-carboxylate oxygens of Glu-111 is 2.7 Å from N(1) of adenine, close enough to form a H-bond if either the former or the latter (as proposed by Wodak et al.) is protonated (Figure 1C). This interaction is also seen in the RNase·dCpA complex [see Wlodawer (1985)]. In contrast, the 4-Å distance between the corresponding atoms in the complex of RNase with an analog of UpA (Richards & Wyckoff, 1973) is too large to allow a strong H-bond, and a molecular dynamics simulation of the RNase·CpA complex shows N(1) of adenine bonding to the side-chain amide nitrogen of Gln-69 rather than to Glu-111 (Brünger et al., 1985). H-bonds between N(6) of adenine and the side-chain carbonyl oxygen atoms of either Asn-67 (Brünger et al., 1985) or Gln-69 and Asn-71 (Wodak et al., 1977) have also been proposed. In theory, minor variations of some of these interactions could still occur with guanine rather than adenine in this site (Figure 1D). Thus, the side-chain amido group of Gln-69 or that of Asn-71 (but not both) may donate a hydrogen to O(6) of guanine (Wodak et al., 1977), and the Glu-111 carboxylate (Wodak et al., 1977) or the side-chain carbonyl oxygen of Gln-69 might accept a hydrogen from N(1) of guanine.

Three of the six proposed base-binding residues of RNase A—Thr-45 and Ser-123 in the B_1 site and Glu-111 in the B_2 site—are conserved in human angiogenin and indeed in all other angiogenins sequenced to date [i.e. bovine, mouse, pig, and rabbit (Maes et al., 1988; Bond & Strydom, 1989; Bond & Vallee, 1990; Bond et al., 1993)]. Thus, the counterparts of these amino acids—Thr-44, Ser-118, and Glu-108— were the sites in angiogenin chosen for mutagenic replacement.

Threonine-44. Three substitutions of Thr-44 were made: Ala, in order to eliminate side-chain H-bonding with minimal environmental disruption; His, in order to increase side-chain size but maintain hydrophilicity and H-bonding capacity; and Asp, in order to potentially eliminate side-chain H-bonding to cytosine but retain the proposed interaction with uracil. We find that the Ala mutation causes a substantial (24–40-fold) decrease in activity toward tRNA and CpN′, demonstrating that Thr-44 is indeed functionally important. This substitution, however, affects the rate of UpN′ cleavage much less markedly, and as a consequence T44A no longer distinguishes between the two pyrimidines at position N. These results support the existence of an H-bond between the Thr β-OH and N(3) of the base and indicate that the bond with cytosine is stronger than that with uracil. They suggest, moreover, that this difference in binding strength may largely account for the 10-fold preference of native angiogenin for cytosine over uracil.

The decreases in k_{cat}/K_m values for CpN′ cleavage accompanying the Thr → Ala mutation correspond to an apparent free energy change of 2.0–2.2 kcal/mol, which is slightly beyond the range of 0.5–1.5 kcal/mol generally observed (Fersht et al., 1985; Street et al., 1986) for deletion of a H-bond between neutral partners. Thus the donor–acceptor distance or the donor–hydrogen–acceptor angle in the native enzyme may be particularly favorable. Alternatively, the small extra free energy difference may derive from secondary effects of the replacement on other interactions.

Mutation of Thr-44 to His reduces activity toward tRNA even more than does the Ala substitution, but with dinucleotides the activity decrease is in fact less than for the Ala mutant. This apparent inconsistency may reflect differences

in assay conditions, e.g., pH or substrate concentration, or the influence of peripheral tRNA components on binding at the B_1 site. As with T44A, the activity changes with CpN′ substrates are much larger than those with UpN′.

The most dramatic specificity change is seen with the T44D derivative, where activity toward CpN′ is 17–40 times lower while that toward UpN′ almost doubles. As a result, T44D prefers uridine over cytidine at position N by a factor of 3–5 and its order of substrate selectivity is UpA > CpA > UpG > CpG, compared to CpA > CpG > UpA > UpG for angiogenin. The activity decrease for CpN′ substrates is similar to that accompanying the Ala substitution and is again consistent with the loss of the proposed H-bond. The increased potency toward UpN′ substrates suggests not only that the side-chain-to-pyrimidine H-bond is maintained in this instance but that the interaction of uracil with Asp makes a greater contribution to binding energy than does that with Thr, likely due to more optimal alignment of bonding groups or the superior H-bonding characteristics of the Asp carboxylate.

Despite its altered specificity, T44D has essentially the same activity as native angiogenin toward tRNA and 18S and 28S rRNA, presumably because the decreases and increases in rates of cleavage of CpN′ and UpN′ sequences, respectively, are balanced. T44D also generates the same limit pattern of 100–500 nucleotide products from the rRNAs as angiogenin, suggesting that the specificity revealed in this pattern may relate to substrate secondary, rather than primary, structure.

The effects of the T44D substitution only partially resemble those observed previously for the corresponding mutation of Thr-45 in rat pancreatic RNase (Miranda, 1990). Both convert an enzyme that prefers cytosine at position N to one that prefers uracil. More detailed comparisons are complicated by the different substrates and assay conditions used to study the two proteins, but the mutation seems to be much more disruptive to RNase than it is to angiogenin. Thus, in contrast to the increased activities of T44D-angiogenin toward UpN′ substrates (pH 5.9, I = 0.05), T45D-RNase has only 0.5% of native activity toward U>p (pH 5.5, I = 0.01).[3] In addition, T45D-RNase has no detectable activity toward C>p pH 6.5 and ~1% activity at pH 5.5 (I = 0.1 in both cases), whereas T44D-angiogenin is 2.5–6% active toward CpN′ substrates at pH 5.9 (I = 0.05).

The activity decreases for hydrolysis of cyclic nucleotides by T45D-RNase correspond to ΔG values of up to several kilocalories per mole, exceeding by far the maximum cited above for the contribution of a H-bond between uncharged moieties. It would therefore appear that the Asp substitution must impair activity by some additional means beyond its effects on the target H-bond, perhaps by sterically hindering pyrimidine binding or by precluding other interactions [e.g., Thr NH to O(2) of the base or Thr carbonyl O to the His-12 imidazole (Richards & Wyckoff, 1973)]. Since all of these factors could apply differentially to cytosine and uracil, it is unclear which of them underlies the change in B_1 site specificity associated with the mutation. In any event, the greater impact of the Thr → Asp substitution on RNase compared to angiogenin suggests different orientations and/or local environments for the threonine residues in the two enzymes. Such differences could be responsible for the increased preference of native angiogenin for cytidine over uridine at position N [10-fold vs 2–3-fold for RNase (Harper & Vallee, 1989)] and may also underlie part of this enzyme's 10^5–10^6-fold lower potency with standard RNase A substrates.[4]

Serine-118. The role of Ser-118 in angiogenin was investigated by mutating it to Asp and to Arg. The Asp carboxylate can serve only as a hydrogen acceptor and therefore cannot bind the uracil carbonyl oxygen, although it might be able to bind the amino group of cytosine. The reverse is true for the Arg guanidino group, an obligate hydrogen *donor*. Thus, if Ser-118 in angiogenin is involved in base recognition in the manner proposed for Ser-123 in RNase, these substitutions would be predicted to alter substrate specificity. We find, however, that both mutations decrease catalytic activity *without* influencing base specificity significantly: S118D has 40–60% of native activity toward tRNA and the four dinucleotides examined, and S118R is 50% active toward UpA and ~25% active toward all of the remaining substrates. These results are consistent with Ser-118 playing a minor, probably indirect, role in catalysis rather than in substrate binding. Alternatively, this residue, unlike its RNase counterpart, may make small, comparable contributions to pyrimidine binding that are eliminated in the mutants, e.g., if the replacement residues are both positioned so as to be incapable of interacting with either base. These possibilities might be distinguished by comparing k_{cat} and K_m values for native vs mutant enzymes; however, as noted above, these individual parameters cannot be measured at this time.

Glutamic Acid-108 and the B_2 Site. The importance of the putative B_2 site residue, Glu-108, was examined by mutating it to Gln and to Lys, residues sharing some of the same H-bonding properties as the original Glu. Gln can serve as either an acceptor or a donor, and can potentially interact with N(1) of either adenine (whether protonated or unprotonated) or guanine. The relative strengths of any such bonds, however, would probably differ considerably from those for the native protein. Lys can act only as a hydrogen donor and therefore cannot substitute for Glu in guanine binding, although it might bind adenine, albeit with different affinity. Thus, if Glu-108 is involved in purine binding as hypothesized for Glu-111 in RNase A, the Gln and Lys replacements would be expected to influence catalytic activity and substrate specificity appreciably. We find, however, that the activities of E108K and E108Q toward RNA and dinucleotide substrates are changed by no more than 50% and that both mutations cause only a slight shift in the preference for adenosine over guanosine at position N′, from ~3-fold with native angiogenin to 5- and 8-fold with E108K and E108Q, respectively. These results indicate that Glu-108 plays no significant role in the enzymatic activity or specificity of angiogenin.

If Glu-108 is not an important B_2 site component, then what is the structural basis for the N′-specificity of angiogenin? The residues thought to constitute the B_2 site of RNase—Asn-67, Gln-69, Asn-71, and Glu-111—are contained on two loops that differ dramatically from their angiogenin counterparts. The Cys-65–Cys-72 disulfide bond that forms one of the loops in RNase is missing from angiogenin and both RNase segments have two more residues than those in angiogenin. Indeed, the only amino acids in this region of RNase that are unambiguously maintained in angiogenin are Glu-111 and the three residues preceding it. Thus, of the three remaining putative B_2 site constituents in RNase, Asn-67 and Gln-69 are not

[3] Activities for T45D-RNase toward cyclic nucleotides are based on k_{cat}/K_m values calculated from data in Table 5.7 of Miranda (1990).

[4] More detailed mechanistic information on the effects of the various Thr-44 mutants, in particular regarding individual steps along the reaction pathway, is not readily obtainable since the anomalous kinetics exhibited by angiogenin (T.P.C. and R.S., unpublished results) thus far preclude determinations of separate k_{cat} and K_m values.

conserved and it is unclear whether Asn-71 has a functional equivalent: the sequences of the two proteins can be aligned so as to imply a correspondence between Asn-68 of angiogenin and Asn-71 of RNase, but the position of Asn-68 in the calculated structure of angiogenin (Palmer et al., 1986) differs from that of Asn-71 in RNase. At the same time, it should be noted that the computational starting point for the angiogenin structure was the X-ray coordinates of RNase and, as a result, the representations of areas of low overall homology, such as this one, may be unreliable. It therefore remains possible that the two aspargines are in fact located similarly in the two proteins. For that matter, other residues in this region of angiogenin also might occupy positions, not apparent from the calculated structure, that are analogous to those of Asn-67, Gln-69, or Asn-71 of RNase, although earlier studies have excluded enzymatic roles for a number of potential candidates, including Arg-70, Asn-109, and the entire segment extending from Asn-61 to Glu-67 (Hallahan et al., 1991,-1992, Shapiro & Vallee, 1992).

An additional consideration is that the conformations of angiogenin-bound dinucleotide substrates may differ markedly from that seen with RNase. Although the effects of various mutations described here and in previous reports (Shapiro & Vallee, 1989; Shapiro et al., 1989) imply that on the whole the pyrimidine nucleoside and the phosphate group are docked to the two enzymes much the same, the orientations of the 5′-nucleotide may be quite different. Thus, the B_2 site of angiogenin may include residues whose participation would not be predicted from the RNase homology. In this regard, the minor change in N′-specificity for the Thr-44 mutants and the more striking change for D116S-angiogenin (Curran & Vallee, 1993) are perhaps significant.

Whatever the identities of the B_2 site residues in angiogenin may be, this recognition site is clearly less selective than that in RNase. Thus, whereas RNase cleaves NpA ~20-fold faster than NpG and several hundred-fold faster than NpU (Witzel & Barnard, 1962), with angiogenin these rates differ by only a factor of 2–3 (Shapiro et al., 1988; Harper & Vallee, 1989) and about 20 (R.S., unpublished results), respectively. Indeed, we have proposed previously (Hallahan et al., 1992) that this region is primarily important for binding to a cell-surface receptor (Badet et al., 1989; Hu et al., 1991) rather than to RNA since mutations of Asn-61, Arg-66, Arg-70, and Asn-109 (Hallahan et al., 1992; Shapiro & Vallee, 1992), deamidations of Asn-61 and Asn-109 (Hallahan et al., 1992), and proteolytic removal of residues 61–67 (Hallahan et al., 1991) all markedly impair angiogenic activity without any substantial effect on enzymatic activity.

Threonine-44 and Angiogenic Activity. The precise series of molecular events initiated by angiogenin that lead to blood vessel formation are unknown at present, as indeed are the detailed mechanisms of action of all other angiogenesis factors (Folkman & Shing, 1992). Previous results nonetheless have indicated that the enzymatic action of angiogenin on some RNA or RNA-like substrate, as yet unidentified, constitutes an essential part of this process. Thus, angiogenic activity is markedly attenuated by disruption of the ribonucleolytic active site of angiogenin by chemical modification of His-13 and His-114 (Shapiro et al., 1986), site-directed mutagenesis of His-13, Lys-40, or His-114 (Shapiro & Vallee, 1989; Shapiro et al., 1989), or binding of placental RNase inhibitor (Shapiro & Vallee, 1987). Moreover, enhancement of the catalytic efficiency of angiogenin by mutation of Asp-116 to His simultaneously increases angiogenicity (Harper & Vallee, 1988). The present findings support and extend this view. T44H, a mutant which is 2–4% active in cleaving polynucleotides, has at least 100 times less angiogenic potency on the CAM than the native protein. T44D, a mutant which is fully active toward RNA substrates, retains native angiogenic activity. These results therefore furnish yet another example of the connection between the two activities of angiogenin, identifying Thr-44 as a residue that plays an important role in angiogenesis through its contribution to ribonucleolytic activity. They also provide a clue as to the nature of the in vivo substrate(s) involved in the induction of new blood vessels by angiogenin. Thus, in view of the undiminished angiogenicity of T44D and its dramatically and selectivity decreased capacity to cleave on the 3′-side of cytosine, the sequence of the physiological target of angiogenin would seem to be UpN′ rather than CpN′.

Finally, a more complete understanding of the molecular basis for the various activity changes, or indeed the absence of changes, observed for the mutant proteins in the present study can best be understood by comparison of their three-dimensional structures with that of angiogenin. The crystallization of native angiogenin and collection of a preliminary X-ray data set have recently been reported (Acharya et al., 1992). Hence, such information should be attainable in the relatively near future.

ACKNOWLEDGMENT

We thank Dr. Bert L. Vallee for continued advice and support, Dr. Daniel J. Strydom for amino acid analyses, and Dr. James W. Fett for CAM assays.

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