Site-Directed Mutagenesis of Histidine-13 and Histidine-114 of Human Angiogenin. Alanine Derivatives Inhibit Angiogenin-Induced Angiogenesis†

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Received April 18, 1989; Revised Manuscript Received June 1, 1989

ABSTRACT: The roles of His-13 and His-114 in the ribonucleolytic and angiogenic activities of human angiogenin have been investigated by site-directed mutagenesis. Replacement of either residue by alanine (H13A and H114A) decreases enzymatic activity toward tRNA by at least 10000-fold and virtually abolishes angiogenic activity in the chick embryo chorioallantoic membrane assay. Both the H13A and H114A mutant proteins compete effectively with angiogenin in the latter assay; only a 5-fold molar excess of H13A over unmodified protein is required for complete inhibition. The His → Ala substitutions, however, do not have any significant effect on the interaction of angiogenin with human placental ribonuclease inhibitor, an extremely potent inhibitor of angiogenin ($K_i \sim 7 \times 10^{-16}$ M) previously shown to interact with another active-site residue, Lys-40. The effects of more conservative replacements—glutamine at position 13 and asparagine at position 114—were also examined. While the enzymatic activity of the H114N mutant was at least 3300-fold less than for the unmodified protein, the H13Q derivative had only 300-fold reduced activity toward tRNA and cytidylyl(3′→5′)adenosine. Both substitutions substantially decreased angiogenic activity. The parallel effects on ribonucleolytic and biological activities observed with all four mutant proteins provide strong evidence that the latter activity of angiogenin is dependent on a functional enzymatic active site. The capacity of the H13A and H114A derivatives to compete with angiogenin in the chorioallantoic membrane assay suggests several additional features of the biological mode of action of this protein.

Human angiogenin, a 14.1-kDa monomeric protein, induces neovascularization in the chick embryo chorioallantoic membrane (CAM)[1] and rabbit cornea (Fett et al., 1985), cleaves RNA (Shapiro et al., 1986b), and exerts a number of effects on vascular endothelial and smooth muscle cells in vitro, which include activating phospholipases C and A_2 (Bicknell & Vallee, 1988, 1989) and increasing cholesterol esterification (Moore & Riordan, 1989). Angiogenin displays extensive sequence homology to the pancreatic RNases (Strydom et al., 1985; Kurachi et al., 1985). Indeed, most of the active-site components of bovine pancreatic RNase A, including the critical catalytic residues His-12, Lys-41, and His-119, are conserved in angiogenin. Nonetheless, the enzymatic activity of angiogenin differs markedly in both magnitude and specificity from that of RNase A (Shapiro et al., 1986b; St. Clair et al., 1987, 1988; Rybak & Vallee, 1988).

Numerous pieces of evidence suggest that the ribonucleolytic and angiogenic activities of angiogenin are related. Both activities are abolished by the human placental RNase inhibitor (PRI) (Shapiro & Vallee, 1987), which binds to angiogenin with a dissociation constant of 7×10^{-16} M (Lee et al., 1989b). Mutation of Lys-40 to glutamine also virtually eliminates both activities (Shapiro et al., 1989). Conversely, a mutation that increases enzymatic activity 15-fold, Asp-116 → His, also increases angiogenicity by 1–2 orders of magnitude (Harper & Vallee, 1988).

Further, chemical modification of histidines in angiogenin by bromoacetate at pH 5.5 decreases both enzymatic and angiogenic activity (Shapiro et al., 1986b, 1987). The sites of carboxymethylation have been identified as His-13 and His-114 (Shapiro et al., 1988b), whose corresponding residues, His-12 and His-119, are modified under these conditions in classical studies on RNase A (Stein & Barnard, 1959; Crestfield et al., 1963). However, unlike with RNase A, it has not been possible to obtain and characterize derivatives of angiogenin carboxymethylated at only a single histidine. For this reason, and because of the inherent limitations and interpretive ambiguities of chemical modification approaches, we have now employed oligonucleotide-directed mutagenesis to investigate the role of these two histidines in angiogenin. Replacement of either histidine by alanine essentially abolishes ribonucleolytic and biological activities. Strikingly, both the H13A[2] and H114A mutants effectively inhibit the angiogenesis induced by angiogenin in the chick embryo CAM assay. The effects of more conservative replacements—i.e., glutamine at position 13 and asparagine at position 114—were also examined.

Experimental Procedures

Angiogenin[3] was obtained from a recombinant expression system in *Escherichia coli* as described (Shapiro et al., 1988a) and was quantitated by amino acid analysis. Bovine pancreatic

† This work was supported by funds from Hoechst, A.G., under an agreement with Harvard University.

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[1] Abbreviations: CAM, chorioallantoic membrane; RNase, ribonuclease; RNase A, bovine pancreatic ribonuclease A; PRI, human placental ribonuclease inhibitor; C18, octadecylsilane; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; CpA, cytidylyl(3′→5′)adenosine; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CpG, cytidylyl(3′→5′)guanosine; C>p, cytidine cyclic 2′,3′-phosphate.

[2] We designate mutant proteins by the single-letter code for the original amino acid followed by its position in the sequence and the single-letter code for the new amino acid.

[3] Angiogenin expressed in *E. coli* differs from natural angiogenin only with respect to its N-terminus: Met-(−1)-Gln-1 versus <Glu-1, respectively. These two forms have indistinguishable enzymatic and angiogenic activities (Shapiro et al., 1988a).

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RNase A and human PRI were obtained and quantitated as described previously (Shapiro et al., 1989).

Oligonucleotide-Directed Mutagenesis. Genes encoding angiogenin with alanine or glutamine replacing His-13 and alanine or asparagine replacing His-114 were obtained by the method of Kunkel (1985) as described (Shapiro et al., 1988a). The mutagenic oligonucleotides were pCTGACCCAGG*CC*TATGACGC (H13A), pCTGACCCAG*CAA*TATGACGCT (H13Q), pTGCCAGTC*GCT*CTAGATCAG (H114A), and pCTGCCAGTC*AAT*CTAGATCAG (H114N). They were purchased from Biotix (Danbury, CT).

Expression and Isolation of Angiogenin Derivatives. Genes encoding mutant angiogenins were transferred from M13mp18 into the expression vector pAng2 by standard procedures (Shapiro et al., 1988a). The expression plasmid contains a modified *E. coli trp* promoter and an ampicillin resistance marker for selection. Expression and purification of recombinant proteins were performed as described (Shapiro et al., 1988a) except that the H13Q and H114N derivatives were rechromatographed on the C18 HPLC column (see Results). In this case, elution was achieved with a gradient of 32–42% solvent B in 45 min at 0.8 mL/min, where solvent A was 0.1% TFA in water and solvent B was 0.08% TFA in a 3:2:2 mixture of 2-propanol/acetonitrile/water. One-minute fractions were collected, lyophilized, and reconstituted in 100 μL of water. All other samples were dialyzed vs water prior to being tested for ribonucleolytic and angiogenic activity. In order to ensure that preparations of angiogenin mutants were not contaminated by unmodified angiogenin, all glassware and centrifuge bottles employed during the isolation were either autoclaved or treated with 0.1 N NaOH for 30 min prior to use. In addition, blank gradients were run on both the Mono S and C18 HPLC columns immediately before each mutant protein was chromatographed. In all cases, the chromatograms revealed no detectable (i.e., <50 ng) carryover of angiogenin from previous use of the columns.

Structural Characterization. Amino acid analyses (Picotag method; Waters Associates) and tryptic peptide mapping were performed as described (Strydom et al., 1985; Shapiro et al., 1988a).

Enzymatic Assays. All buffers and water used in enzymatic assays were passed through a Sep-Pak C18 cartridge (Waters) to remove adventitious RNases. Formation of perchloric acid soluble fragments from yeast tRNA (type X, Sigma Chemical Co.) was measured as described (Shapiro et al., 1987). Incubation mixtures contained 0.6 mg of RNA, 30 μg of human serum albumin, 33 mM Hepes, and 33 mM NaCl, pH 6.8, in a 300-μL volume. Maximum concentrations of mutant angiogenins were 10.4 μM (H13A), 6.6 μM (H13Q), 5.0 μM (H114A), and 7.5 μM (H114N), and incubation times of 4–7 h at 37 °C were employed. For comparison of nonenzymatic and angiogenin-catalyzed cleavage rates, the assay was modified in that albumin was omitted and the tRNA was purified to remove extraneous RNases. The tRNA stock solution (10 mg/mL in water) was extracted with phenol, with a 1:1 mixture of phenol and chloroform, and then with chloroform 3 times. One-tenth volume of 3 M sodium acetate, pH 5.2, was then added, followed by 2.5 volumes of ethanol. After centrifugation (15600*g*, 15 min, 4 °C), the RNA pellet was washed with 70% ethanol, dried, and reconstituted in water. For the assays measuring the nonenzymatic rate of tRNA cleavage, a 5-h incubation at 37 °C in the mixture described above was employed. For comparison, a sample containing angiogenin (0.24 μM) was incubated for 30 min at 37 °C. The absorbances at 260 nm of the perchloric acid soluble supernatants were compared to those produced by unincubated control samples lacking angiogenin.

Table I: Amino Acid Compositions of Angiogenin Mutants Produced in *E. coli*[a]

		mutants			
amino acid	angiogenin[b]	H13A	H13Q	H114A	H114N
Asx	14.9 (15)	15.0	14.9	14.6	16.1
Glx	10.0 (10)	10.0	10.7	10.0	9.8
Ser	8.1 (9)	8.1	7.9	8.5	8.2
Gly	8.3 (8)	8.1	8.3	8.2	8.1
His	5.9 (6)	4.9	4.8	4.9	5.0
Arg	13.0 (13)	12.9	12.8	12.9	13.3
Thr	7.2 (7)	7.1	6.8	7.0	6.8
Ala	5.1 (5)	6.1	5.1	6.0	5.1
Pro	8.1 (8)	8.0	8.1	8.3	8.2
Tyr	3.7 (4)	3.9	3.5	3.9	3.6
Val	4.2 (5)	4.2	4.1	4.2	4.2
Met	2.0 (2)	2.0	2.0	2.0	2.1
Ile	6.7 (7)	6.7	6.5	6.6	6.5
Leu	6.1 (6)	6.0	6.5	6.1	6.1
Phe	5.0 (5)	5.0	5.0	4.9	4.8
Lys	6.7 (7)	6.8	7.4	7.2	7.0
Cys	5.1 (6)	5.2	5.1	5.2	5.1

[a] Analyses were performed in duplicate as described (Strydom et al., 1985). Tryptophan contents were not determined. [b] Values in parentheses from sequence [plus Met-(−1) residue].

Cleavage of the dinucleotide CpA (100 μM) by angiogenin and H13Q-angiogenin in 33 mM Mes/33 mM NaCl, pH 5.8, was measured by HPLC as described (Shapiro et al., 1986a, 1988a). With the H13Q derivative (12.8 μM), an incubation time of 20 h at 37 °C was employed. In order to measure the nonenzymatic rate of cleavage, a sample of 1 mM CpA in the same buffer was incubated at 37 °C for up to 11 days.

Biological Assays. Angiogenic activity was assayed by the chick embryo CAM method of Knighton et al. (1977) as described (Fett et al., 1985). The repeatability and reproducibility of this assay have been documented previously (Fett et al., 1985). The number of eggs employed in any individual group of assays at a given concentration ranged from 7 to 17.

Interaction of Angiogenin Derivatives with Human PRI. Apparent second-order rate constants for association of PRI with angiogenin derivatives were determined by examining their competition with RNase A for PRI as described (Lee et al., 1989a; Lee & Vallee, 1989a; Shapiro et al., 1989). Rate constants for dissociation of angiogenin derivatives from their complexes with PRI were also measured by methods reported previously (Lee et al., 1989b). The conditions employed were identical with those described in Shapiro et al. (1989).

Results

Preparation of Angiogenin Mutants. Genes encoding H13A-, H13Q-, H114A-, and H114N-angiogenin were prepared by oligonucleotide-directed mutagenesis as described previously (Shapiro et al., 1989). The DNA for each mutant was sequenced in its entirety in order to rule out any spurious mutations. Mutant proteins were expressed under the control of the *trp* promoter in *E. coli* strain W3110 and purified as described (Shapiro et al., 1988a). All derivatives had elution times indistinguishable from those of unmodified angiogenin during both Mono S and C18 HPLC. The final preparations were >98% pure as judged by SDS–PAGE (not shown). Yields were 0.5 mg/L (H13Q) to 2 mg/L (H13A).

Structural Characterization. Amino acid compositions of the four mutant angiogenins (Table I) support the proposed structures and indicate that all proteins contain a Met-(−1) residue. Tryptic peptide maps (H13A and H114A shown in Figure 1) indicate that in all cases the three disulfide bonds

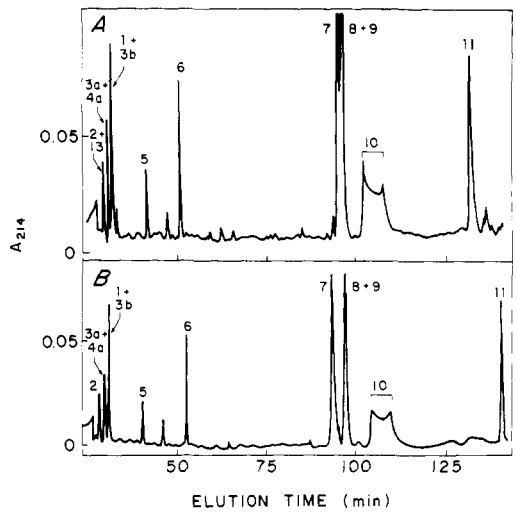

FIGURE 1: Chromatography of tryptic digests of 3.5 nmol of H13A-angiogenin (panel A) and 3.7 nmol of H114A-angiogenin (panel B) on a C18 HPLC column.

Table II: Amino Acid Compositions of Tryptic Peptides from Angiogenin Mutants[a]

amino acid	H13A T7[b]	H13Q T7	H114A T11	H114N T11
Asx	0.93 (1)	1.09 (1)	4.00 (4)	4.91 (5)
Glx	1.86 (2)	2.79 (3)	2.99 (3)	2.87 (3)
Ser			1.03 (1)	0.95 (1)
Gly	1.11 (1)	1.32 (1)	1.17 (1)	1.56 (1)
His	1.05 (1)	0.99 (1)		0.10
Arg	0.89 (1)	1.09 (1)	1.03 (1)	1.06 (1)
Thr	1.93 (2)	1.87 (2)		
Ala	1.95 (2)	0.98 (1)	2.89 (3)	1.93 (2)
Pro	1.07 (1)	1.07 (1)	1.19 (1)	1.20 (1)
Tyr	1.80 (2)	1.81 (2)		
Val			2.95 (4)	2.96 (4)
Met				
Ile			1.70 (2)	1.79 (2)
Leu	0.98 (1)	1.04 (1)	2.03 (2)	2.16 (2)
Phe	0.96 (1)	0.92 (1)	0.82 (1)	0.91 (1)
Lys	1.03 (1)	0.91 (1)	0.82 (1)	0.94 (1)
pmol analyzed	175	224	90	240

[a] Cystine and tryptophan contents were not determined. Numbers in parentheses represent compositions of the mutant proteins expected for peptide T7 (amino acids 6–21) or peptide T11 (disulfide-linked amino acids 55–60 + 102–121). [b] Peptide T7 of H13A-angiogenin coelutes with peptides T8 and T9 during C18 HPLC (Figure 1). The amino acid composition of peptide T7 shown has been corrected for the presence of 0.22 molar equiv of T8 and 0.15 equiv of T9.

(in peptides T9, T10, and T11) formed correctly [see Strydom et al. (1985) for a description of angiogenin tryptic peptides]. For the H13A- and H13Q-angiogenins, amino acid compositions of peptide T7 (Table II), containing residue 13, indicate the replacement of His by Ala or Glx, respectively, and no other changes. Peptide T7 of the H13A mutant elutes later than the natural peptide T7 during C18 HPLC, as expected for replacement of His by Ala. For the H114A- and H114N-angiogenins, amino acid compositions of peptide T11 (Table II), containing residue 114, show the replacement of His by Ala or Asx, respectively, and no other changes. The H114A peptide elutes somewhat later than the corresponding peptide from angiogenin, again consistent with the His → Ala replacement. Amino acid compositions of the remaining peptides are in good agreement with the proposed structures and account for the rest of the four proteins, except for Arg-32 and Arg-33 of H114A.

Ribonucleolytic Activity of Angiogenin Derivatives. No enzymatic activity toward tRNA could be detected with the H13A- and H114A-angiogenins, allowing an upper limit to be set at 0.01% activity compared with unmodified angiogenin. Initial preparations of the H13Q and H114N derivatives, however, appeared to be 0.1–1% active. In order to examine whether this activity might be due to trace amounts of a contaminating ribonuclease, both mutant proteins were rechromatographed on a C18 HPLC column employing a shallow gradient (see Experimental Procedures). Fractions collected from across each peak were lyophilized, reconstituted in water, quantitated by amino acid analysis, and tested for enzymatic activity. With the H13Q derivative, the three major protein-containing fractions had similar specific activities, 0.3% that of native angiogenin. Thus, this activity appears to be associated with the mutant protein. With the H114N derivative, the specific activity varied considerably through the peak. The earliest fractions had no measurable activity, under conditions where an activity 0.03% of that of angiogenin could have been detected. This value thus represents an upper limit on the true activity.

Activity of the rechromatographed H13Q mutant toward CpA was also measured. A k_{cat}/K_m value of 0.045 M^{-1} s^{-1} was obtained, 0.4% of that determined for angiogenin. Owing to limitations of the assay, it has not been possible to determine whether this activity decrease reflects changes in k_{cat}, K_m, or both.

Rate of Nonenzymatic Cleavage of tRNA and CpA. In order to facilitate mechanistic evaluation of ribonucleolytic activities measured with mutant angiogenins, the nonenzymatic rates of substrate cleavage were examined. At pH 6.8, 37 °C, the observed reaction velocity for formation of perchloric acid soluble fragments from tRNA was 3.3×10^{-4} A_{260} units/min at a substrate concentration of 2 mg/mL. The initial reaction velocity with 0.24 μM angiogenin and 2 mg/mL tRNA was 1.2×10^{-2} A_{260} units/min. In calculation of the difference between the rate constant for the nonenzymatic reaction ($=v_0/[S]$) and that for the angiogenin-catalyzed reaction ($=V_{max}/[E]$) from these data, the following assumptions were made: (a) all phosphodiester bonds in the substrate are equally susceptible to nonenzymatic cleavage; (b) no more than 10% of these bonds are attacked by angiogenin in the initial stages of the reaction [see Rybak and Vallee (1988)]; and (c) the catalyzed reaction rate is approximately half of V_{max} (Lee & Vallee, 1989b). On this basis, the calculated rate enhancement is at least 10^5.

The rate enhancement by angiogenin was also examined in a less complex system, employing the dinucleotide CpA as substrate. After 11 days at 37 °C, pH 5.8, in the absence of angiogenin, <0.05% conversion to products were observed by HPLC. Thus, the pseudo-first-order rate constant for the reaction is $<5 \times 10^{-10}$ s^{-1}. Under the same conditions, k_{cat} for angiogenin is ~0.1 s^{-1} (T. P. Curran and R. Shapiro, unpublished results). Thus, the overall rate enhancement is $>2 \times 10^8$.

Biological Activity of Angiogenin Derivatives. The angiogenic activities of the H13A-, H114A-, H13Q-, and H114N-angiogenins were measured in the CAM assay at doses ranging from 1 to 10 ng per egg (Table III). In all cases, activities are substantially decreased compared with that of angiogenin. Thus, the percentage of positive responses at 10 ng/egg for each mutant (17–25%) is considerably lower than the 47% observed for unmodified angiogenin at 1 ng/egg, and not significantly different from typical values observed with control samples lacking angiogenin (see legend of Table III).

Angiogenesis Inhibition by Mutant Angiogenins. The capacity of the H13A- and H114A-angiogenins to inhibit ang-

Table III: Angiogenic Activity of Angiogenin and Angiogenin Mutants[a]

sample	dose (ng)	% positives (total no. of eggs)
angiogenin	10	58 (19)
	5	47 (19)
	1	47 (17)
H13A-angiogenin	10	22 (18)
	5	18 (17)
	1	26 (19)
H114A-angiogenin	10	25 (20)
	5	29 (17)
	1	19 (16)
H13Q-angiogenin	10	22 (23)
H114N-angiogenin	10	17 (18)

[a] The CAM assay was employed (Knighton et al., 1977; Fett et al., 1985). Data from two experiments (7–12 eggs/group) did not differ significantly and were combined. Control samples containing only water assayed during the same period of time on 104 eggs were 12% positive.

Table IV: Inhibition of Angiogenic Activity by Angiogenin Mutants[a]

sample	dose (ng)	% positives (total no. of eggs)	range (%)
angiogenin	1	52 (115)	47–60
H114A-angiogenin	20	24 (59)	13–31
angiogenin + H114A-angiogenin	1 + 20	27 (52)	18–43
H13A-angiogenin	20	22 (59)	13–27
angiogenin + H13A-angiogenin	1 + 1	37 (30)	33–40
angiogenin + H13A-angiogenin	1 + 5	20 (30)	19–21
angiogenin + H13A-angiogenin	1 + 20	23 (52)	9–33

[a] The CAM assay was employed. Data from multiple experiments (11–17 eggs per group) were combined. Control samples containing only water assayed during the same period of time on 104 eggs were 12% positive.

iogenin-induced angiogenesis was examined. The amount of unmodified angiogenin used in these experiments, 1 ng, is in that region of the dose–response curve where activity decreases would be most readily apparent (Fett et al., 1985; Kurachi et al., 1988); it was sufficient to induce positive responses on 52% of the eggs. In the presence of 20 ng of the H13A- or H114A derivatives, the percentages of positive responses to 1 ng of angiogenin were only 23% and 27%, respectively (Table IV). These are essentially the same as observed for 20 ng of each mutant protein alone (22 and 24%, respectively). This inhibitory effect was examined further with the H13A derivative, in this case employing 1 and 5 ng of mutant protein together with 1 ng of unmodified angiogenin (Table IV). With 5 ng of H13A-angiogenin, the inhibition was as effective as with 20 ng. Even with only 1 ng, partial inhibition was observed.

Interaction of H13A- and H114A-Angiogenin with Placental Ribonuclease Inhibitor. The apparent second-order rate constants of association for H13A- and H114A-angiogenin with PRI were determined by using a previously described competition assay (Lee et al., 1989a; Shapiro et al., 1989). RNase A (4.5 nM) and angiogenin derivatives (3.7–10.4 nM) were mixed, PRI (4.5 nM) was added, and then the concentration of free RNase A was quantitated by adding the dinucleotide CpG and measuring its rate of cleavage spectrophotometrically. This procedure yielded rate constants of $(4.1 \pm 0.7) \times 10^8$ M^{-1} s^{-1} for H13A and $(2.2 \pm 0.4) \times 10^8$ M^{-1} s^{-1} for H114A, compared with $(4.0 \pm 0.3) \times 10^8$ M^{-1} s^{-1} for unmodified Met-(−1) angiogenin (Shapiro et al., 1989).

The rate constants for dissociation of the complexes of PRI with H13A- and H114A-angiogenin were measured by first forming each complex, then adding a 250-fold excess of RNase A as scavenger for free PRI, and measuring the release of free angiogenin derivative by cation-exchange HPLC (Figure 2). In both cases, the dissociation was biphasic with 12–13% of the angiogenin released during the first 2 days, followed by dissociation of an additional 5% (H13A) or 13% (H114A) during the next 8 days.[4] Treating the second, slower, phase as a first-order process, dissociation rate constants of 8.1×10^{-8} and 2.2×10^{-7} s^{-1} were calculated for H13A and H114A, respectively. The corresponding value for native Met-(−1)-angiogenin determined at the same time was 1.5×10^{-7} s^{-1}.

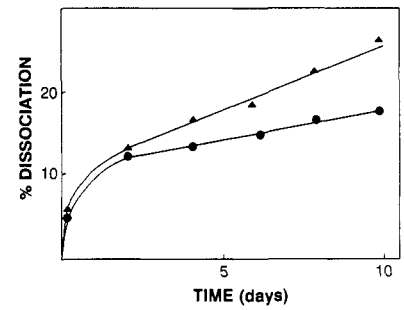

FIGURE 2: Dissociation of H13A-angiogenin·PRI (circles) and H114A-angiogenin·PRI complexes (triangles). Angiogenin derivatives were incubated with 1.5 equiv of PRI for 20 min at 25 °C, followed by addition of 250 equiv of RNase A. Release of free angiogenin was then measured by cation-exchange HPLC.

The K_i values calculated from these association and dissociation rate constants are 3.3×10^{-16}, 2.0×10^{-16}, and 1.0×10^{-15} M for unmodified and H13A- and H114A-angiogenins, respectively.

Discussion

Critical functional roles for His-12 and His-119 of RNase A were originally proposed on the basis of what are now classical chemical modification studies (Gundlach et al., 1959; Stein & Barnard, 1959; Crestfield et al., 1963; Heinrikson et al., 1965). Treatment of RNase with either iodo- or bromoacetate at pH 5.5 carboxymethylated $N\delta_1$ of His-119 and $N\epsilon_2$ of His-12.[5] Homogeneous derivatives modified at one or the other of these sites were isolated and shown to be inactive. Numerous additional reagents have since been demonstrated to inactivate the enzyme through reaction at these sites also, including iodoacetamide (Fruchter & Crestfield, 1967), 2′-(3′)-*O*-bromoacetyluridine (Pincus et al., 1975), and ferrate ion (Steczko et al., 1979). The view of His-12 and -119 as important catalytic residues has been supported by a multitude of X-ray and neutron diffraction studies of RNase and its complexes with inhibitors (Richards & Wyckoff, 1971, 1973; Wlodawer & Sjolin, 1981; Wlodawer et al., 1983, 1988; Borkakoti, 1983; Campbell & Petsko, 1987). Nonetheless, the essentiality of these two histidines is not as yet established rigorously, and the postulated role of His-12 has been questioned (Machuga & Klapper, 1975; Deakyne & Allen, 1979).

The enzymatic activity of angiogenin is lost upon treatment with bromoacetate at pH 5.5, albeit 5-fold more slowly than is that of RNase A (Shapiro et al., 1987). The primary sites

[4] A similar biphasic process, with relatively rapid dissociation of a small percentage of the complex followed by slower dissociation of the remainder, has been observed previously for the complexes of PRI with RNase A and, to a lesser extent, angiogenin (Lee et al., 1989b). It has also been reported for other systems, e.g., complexes of avidin with biotin derivatives (Garlick & Giese, 1988).

[5] The system of Oldfield et al. (1975) is used when referring to the side chain atoms of amino acids.

of modification are $N\delta_1$ of His-114 and $N\epsilon_2$ of His-13, with the former reacting ~1.5-fold more rapidly than the latter (Shapiro et al., 1988b). By comparison, in RNase A His-119 reacts 8-fold faster than His-12 (Crestfield et al., 1963). The capacity of angiogenin to induce neovascularization on the CAM also appears to be markedly diminished by carboxymethylation (Shapiro et al., 1986b). Thus, these experiments point to critical roles for the two residues in both the enzymatic and biological activities of this protein. In addition, the carboxymethyl derivatives have been shown to bind placental RNase inhibitor 15-fold less tightly than does unmodified angiogenin, suggesting that the two histidines are part of the contact region (Lee & Vallee, 1989a).

Unambiguous interpretation of the chemical modification results is limited by several factors. Since an additional substituent has been attached to the imidazole ring, it is impossible to attribute the activity decrease solely to alteration of that functional group. This is further complicated by the anionic nature of the substituent, since the substrate is also anionic. In addition, it has not been possible to isolate an angiogenin derivative modified at only one site and determine whether it is completely devoid of activity.

In principle, these limitations can be largely circumvented by employing site-directed mutagenesis. By this method, the imidazole moiety can be removed, as in the Ala derivatives, or conservatively replaced, as in the Gln and Asn derivatives. Characterization of the His → Ala mutants allows the essentiality of these residues for ribonucleolytic, angiogenic, and other activities to be assessed. Additional functional information can then be obtained by studying the His → Gln and His → Asn mutants. These replacements could potentially provide some of the same hydrogen-bonding interactions as the original histidine (Lowe et al., 1985). In RNase A, $N\epsilon_2$ of His-12 and $N\delta_1$ of His-119 are thought to participate in catalysis (see below). Since bromoacetate reacts with the same imidazole nitrogens (on the corresponding residues) in angiogenin as in RNase A, it is probable that the same nitrogens are involved in catalysis. Thus, for His-13 of angiogenin, glutamine was selected as the most conservative substitution since its ϵ-NH_2 occupies a position analogous to that of the imidazole $N\epsilon_2$. His-114 was replaced by an asparagine since the asparagine δ-NH_2 can hydrogen bond in a manner similar to the imidazole $N\delta_1$.

We find that the H13A, H114A, and H114N mutants have no detectable enzymatic activity. In contrast, the H13Q mutant is only ~300-fold less active than angiogenin toward both tRNA and CpA. It is most likely that this activity is due to the mutant itself rather than a contaminant for several reasons. Contamination by a ribonuclease is improbable since enzymatic activity and H13Q-angiogenin comigrate during C18 HPLC. In addition, the similar levels of activity observed toward tRNA and CpA indicate an angiogenin-like specificity, markedly different from those of typical bacterial and mammalian RNases (Barnard, 1969, and see below). Contamination by unmodified angiogenin is also unlikely in view of the precautions described under Experimental Procedures. Finally, similar activity was measured in two different preparations.

Any interpretation of the activity decreases accompanying mutations of angiogenin should be made in light of the overall rate enhancement achieved in angiogenin-catalyzed reactions. Angiogenin cleaves tRNA and CpA ($\sim 5 \times 10^4$)-fold and $\sim 10^6$-fold, more slowly, respectively, than does RNase A under the conditions employed in the present study (Lee & Vallee, 1989b; Harper & Vallee, 1989).[6] The magnitude of the enhancement of C>p hydrolysis by RNase A is reported to be $\sim 7 \times 10^9$ (Eftink & Biltonen, 1983). The enhancement of the initial transphosphorylation step would be expected to be much higher (Eftink & Biltonen, 1987), but no value appears to have been reported. We find that the pseudo-first-order rate constants for the nonenzymatic cleavage of tRNA and CpA are at least 10^5-fold and (2×10^8)-fold lower, respectively, than the apparent k_{cat} values for the angiogenin-catalyzed reactions. These represent lower limits since part of the "non-enzymatic" rate may be due to trace amounts of RNase, despite the precautions taken to avoid this, and since the mechanisms and/or rate-limiting steps for the nonenzymatic and angiogenin-catalyzed reactions may differ.

The rate decreases accompanying mutation of His-13 and His-114—$\geq 10^4$-fold for His-13 → Ala and His-114 → Ala and $\geq 10^{3.5}$-fold for His-114 → Asn, but only 300-fold for His-13 → Gln—may have important implications for the catalytic mechanisms of this enzyme and the homologous RNases. The most widely accepted mechanism for RNase A is that originally proposed by Findlay et al. (1962) and subsequently extended by Roberts et al. (1969). In the transphosphorylation step of this mechanism (Figure 3), the primary role of His-12 is to act as a general base catalyst, removing a proton from the ribose 2′-hydroxyl group and thereby facilitating attack on the phosphorus to form a pentacovalent transition state. This species then breaks down to yield the 2′,3′-cyclic phosphate, aided by His-119, which acts as a general acid, donating a proton to the leaving group. The results of most chemical, NMR, and X-ray diffraction studies [see Richards and Wyckoff (1971, 1973), Blackburn and Moore (1982), and Eftink and Biltonen (1987)] support this view and point to the involvement of $N\epsilon_2$ of His-12 and $N\delta_1$ of His-119.

The inactivity of the H13A-, H114A-, and H114N-angiogenins is consistent with this catalytic mechanism. The observed activity of the H13Q derivative suggests that even partial deprotonation of the 2′-hydroxyl by a residue at this position can impart a substantial rate enhancement, in agreement with a theoretical study of RNase by Deakyne and Allen (1979). These authors note that a hydrogen bond between $N\delta_1$ of His-12 and the carbonyl of Thr-45 may prevent the 2′-hydroxyl from becoming totally deprotonated through interaction with $N\epsilon_2$. Instead, a bent hydrogen bond between $N\epsilon_2$ and the 2′-hydroxyl would increase the negative charge on $O_{2'}$, activating it for attack on the phosphorus. In this case, it is reasonable that Gln-13 angiogenin would also be partially active, since it too might form a hydrogen bond with $O_{2'}$. Alternatively, the 300-fold decreased activity of H13Q-angiogenin could reflect the difference between total and partial deprotonation of the 2′-hydroxyl.

Results of a joint X-ray and neutron diffraction analysis of RNase A (Wlodawer et al., 1983) have suggested a different possible role for His-12 that is also consistent with the relatively modest activity decrease accompanying the His → Gln replacement in angiogenin. In this study of the complex of RNase A with uridine vanadate, a putative transition-state analogue, His-12 was observed to hydrogen bond to one of the equatorial oxygens of vanadate rather than to the apical 2′-oxygen. Instead, Lys-41 hydrogen bonds to the 2′-oxygen. This interaction of Lys-41 has also been suggested by NMR

[6] In contrast, angiogenin cleaves specific bonds of rRNA in the intact ribosome somewhat more quickly than does RNase (St. Clair et al., 1987). It is possible that other substrates, as yet undiscovered, are cleaved by angiogenin at rates comparable to those observed for RNase A acting on its best substrates (e.g., CpA).

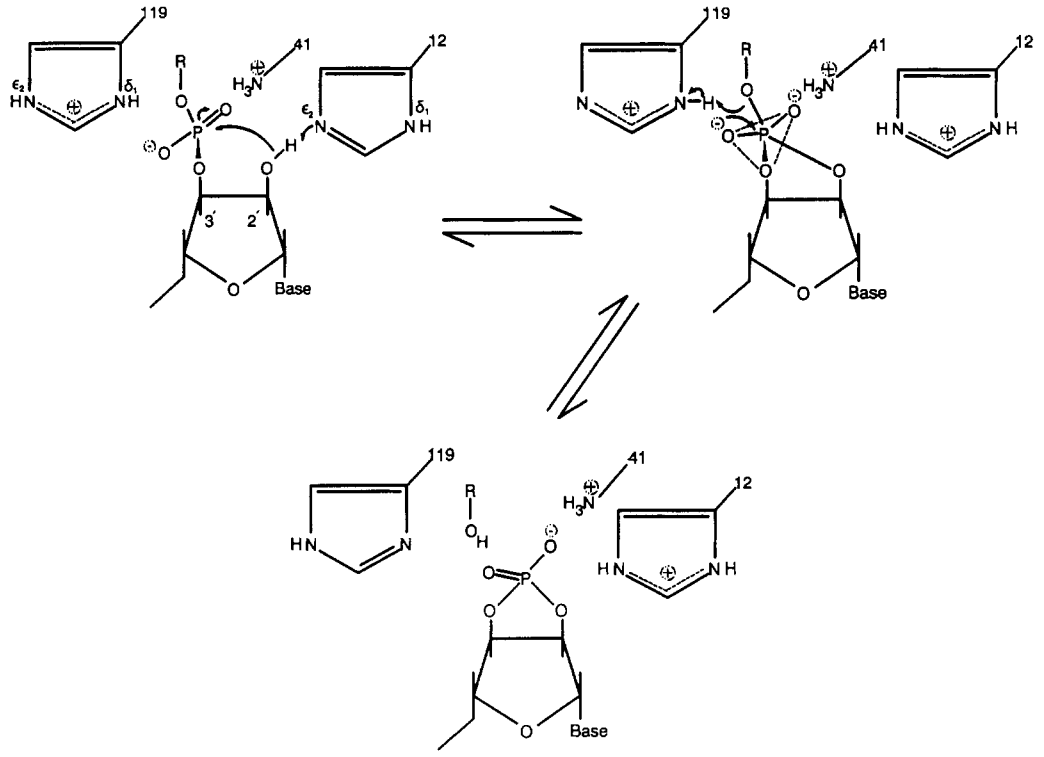

FIGURE 3: Proposed mechanism for the transphosphorylation reaction catalyzed by RNase A, adapted from Roberts et al. (1969).

of the ^{13}C-methylated enzyme (Jentoft et al., 1981). If this view of His-12 is correct [this depends in part on whether uridine vanadate represents a true transition-state analogue (Wlodawer et al., 1983)], then glutamine might well be able to fulfill in part the function of the histidine.

Clearly, direct extension to RNase A of the present results for angiogenin may be unwarranted. As stated above, angiogenin is several orders of magnitude less active than RNase A toward the substrates used in this study. The molecular basis for this difference is unknown but could reflect changes in substrate binding interactions, reactivity of catalytic residues, or even reaction mechanism.

Previous work has indicated that human PRI binds to the active site of angiogenin. Thus, replacement of Lys-40 by glutamine or arginine decreases binding affinity by 1300-fold (Lee & Vallee, 1989a) or 100-fold (Shapiro et al., 1989), respectively. In addition, PRI is a competitive inhibitor of RNase A (Lee et al., 1989b) and therefore, presumably, of angiogenin. Measurement of the interaction of PRI with a mixture of angiogenin derivatives carboxymethylated at either His-13 or His-114 revealed a 15-fold increase in K_i (Lee & Vallee, 1989a), suggesting that these residues may also contact the inhibitor. The present study, however, demonstrates that neither histidine participates in a major interaction with PRI since the H13A derivative binds with a K_i value slightly lower than that obtained with unmodified angiogenin and the K_i value for the H114A derivative is only ~3-fold higher. Thus, the substantial increase resulting from carboxymethylation may have reflected interference by the negative carboxymethyl groups.

One of the most compelling reasons for preparing and studying mutants of angiogenin is to gain insight into its biological action. In this regard, the relationship between the angiogenic and ribonucleolytic activities of angiogenin has been a critical issue. Previous studies have strongly suggested that the former activity is dependent on the latter. Thus, both activities are abolished by binding of PRI (Shapiro & Vallee, 1987) and mutation of Lys-40 to glutamine (Shapiro et al., 1989). Replacement of Asp-116 by histidine increases both activities by more than 1 order of magnitude (Harper & Vallee, 1988). The His-13 and His-114 mutants now provide further evidence for this dependence, since they also show parallel decreases in angiogenic and enzymatic activities. These results indicate, at the least, that a functional active site is necessary for angiogenic activity. Since numerous other ribonucleolytic enzymes, including bovine RNase A, a human tumor cell derived RNase (Shapiro et al., 1986a), and eosinophil-derived neurotoxin, are not angiogenic (Fett et al., 1985; J. W. Fett, personal communication), it is clear that RNase activity per se does not confer angiogenicity. Whether the specific enzymatic action characteristic of angiogenin is sufficient to induce neovascularization remains an open question.

The observed inhibition of angiogenin-induced angiogenesis by the H13A and H114A mutants suggests several further features of the biological mode of action of angiogenin. Inhibition presumably reflects successful competition of the mutant over the unmodified protein for some target. This is most easily understood in terms of specific interaction with target cells, e.g., via a receptor, rather than with a diffusible substance which angiogenin might convert to an angiogenic product. (If such an extracellular substrate were involved, it would have to be present at concentrations similar to or lower than that of angiogenin itself in order for inhibition to be observed.) Recent studies have shown that angiogenin does indeed act directly on vascular endothelial and smooth muscle cells in culture, rapidly eliciting a variety of transient responses. These include activation of phospholipase C, resulting in an increase in 1,2-diacylglycerol levels (Bicknell & Vallee, 1988; Moore & Riordan, 1989), and activation of phospholipase A_2, thereby stimulating prostacyclin secretion (Bicknell & Vallee, 1989). It has not yet been determined whether there is a causal relationship between these cellular effects and angiogenesis. However, preliminary results (R. Bicknell, personal communication; Fox & Riordan, 1989) with the H13A, H114A, and other mutants suggest that alterations in responses in vivo and in vitro closely parallel one another.

Angiogenesis inhibition by the His → Ala mutants clearly indicates that binding of angiogenin to the appropriate receptor or target molecule is not sufficient to induce angiogenesis. Either the catalytic action of angiogenin is required or else binding of angiogenin to its target must induce a conformational or chemical change that in some other manner depends on both histidines. These two possibilities cannot be distinguished at present. The fact that independent mutations of four active-site residues—His-13, Lys-40, His-114, and Asp-116—affect enzymatic and angiogenic activities similarly is perhaps more readily understood if an actual catalytic event is involved.

The results of the competition experiments also indicate that neither His-13 nor His-114 is involved in an interaction that is critical in terms of the affinity of angiogenin for its target. Thus, even an equimolar amount of H13A-angiogenin is sufficient to cause partial inhibition, and a relatively small (5–20-fold) excess of either mutant inhibits completely.

A major goal of angiogenin research has been the development of antagonists that may inhibit undesirable vascular proliferation, such as in growth of solid tumors or neovascular glaucoma (Vallee et al., 1985; Folkman & Klagsbrun, 1987). The homology between angiogenin and pancreatic RNase suggested that PRI might bind tightly to angiogenin, and PRI was indeed shown to inhibit angiogenesis on the chick embryo CAM (Shapiro & Vallee, 1987). A number of factors, however, may limit the therapeutic utility of this inhibitor, including its sensitivity to oxidation and inactivation by metals (Blackburn et al., 1977) and its apparent tight binding to all members of the alkaline RNase family, which are abundant in most body compartments.

The H13A and H114A mutant angiogenins provide an alternative type of antagonist. Since these derivatives appear to compete with angiogenin for its target, they should have much greater specificity. In addition, they should be relatively stable. Further, it may be possible to obtain, either fortuitously or by design, inactive mutants that will bind much more effectively than angiogenin itself.

Finally, we note that the conclusions drawn above based on the inactivity of angiogenin mutants are to some extent restricted by uncertainties regarding the three-dimensional structures of these proteins. Ideally, it would be desirable to establish that the only difference in structure between unmodified and mutant proteins is at the position of the side chain which is altered [see Gerlt (1987) and Knowles (1987) for discussion of this issue]. X-ray crystallography has indicated that this is the case for several proteins [e.g., see Howell et al. (1986)]. However, there is now at least one well-documented instance, involving staphylococcal nuclease (Hibler et al., 1987; Wilde et al., 1988), in which minor changes in active-site residues have produced unexpected conformational changes as far away as 15–30 Å. It is not known whether these effects influence activity.

The following pieces of evidence, taken together, strongly suggest that for the angiogenin mutants under consideration significant conformational changes are absent. First, the three disulfide bonds, involving widely separated regions of the protein, have formed correctly (Figure 1). The existence of these disulfides greatly limits the extent of any possible conformational changes. It may be noted that staphylococcal nuclease, the protein in which such effects do occur, contains 149 amino acids and no disulfide bonds. Second, the Mono S and C18 HPLC elution times for both mutants are indistinguishable from those of angiogenin [see Shapiro et al. (1989)]. Third, the extremely tight binding of PRI, known to involve the active site of angiogenin, is virtually unaffected by the two mutations in question. Fourth, the inactive H13A and H114A mutants compete effectively with angiogenin in the CAM assay, indicating a lack of significant structural perturbations in that region of the protein responsible for binding to its target. Incontrovertible proof, however, must await the determination of the three-dimensional structure of angiogenin and its mutant derivatives by crystallography or NMR. In any event, these considerations have no bearing on either the potential therapeutic utility of the H13A and H114A mutants or the biological implications of their anti-angiogenic capacity.

Acknowledgments

We thank Dr. James F. Riordan for valuable advice and discussions, Dr. Daniel J. Strydom for amino acid analyses, and Christopher Lee and Nicholas Komar for excellent technical assistance.

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