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Ribonucleolytic activity of angiogenin: Essential histidine, lysine, and arginine residues

(bovine pancreatic RNase A/protein homology)

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ABSTRACT **The homology of angiogenin and pancreatic RNase A provides a compelling reason to systematically compare the characteristics of the two proteins using the chemical modification approaches that proved essential to understanding the action of RNase. Reagents specific for histidine, lysine, and arginine markedly decrease the ribonucleolytic activity of angiogenin, much as has been observed for RNase A. Activity is abolished by reduction of the disulfide bonds and is restored by reoxidation. Methionine, tyrosine, and carboxyl group reagents have no significant effect. From the point of view of reactivity, the histidine and lysine residues in angiogenin are severalfold less susceptible to modification than those in RNase A. Arginine reagents, on the other hand, inactivate angiogenin considerably faster than RNase A. Considering specificity, bromoacetate inactivates angiogenin at pH 5.5 by modifying 1.5 histidines, but lysine and arginine reagents are less specific. Thus, 3.8 and 6.3 residues, respectively, are modified by 1-fluoro-2,4-dinitrobenzene and by formaldehyde plus cyanoborohydride, under conditions where activity decreases by ≈80% in both cases. With phenylglyoxal, 6.7 arginines are lost when there is 92% inactivation. Poly(G) prevents inactivation by lysine and arginine reagents, and phosphate protects against the effects of lysine modification. Thus, the functional consequences of these modifications likely reflect the loss of critical residues rather than general conformational effects.**

The blood vessel-inducing protein angiogenin (1) displays extensive amino acid sequence similarity to the pancreatic RNases (2, 3). The recognition of this remarkable homology resulted in the finding that angiogenin possesses ribonucleolytic activity, which is quite distinct from that of pancreatic RNase (4). Furthermore, it led to the discovery that the human placental RNase inhibitor binds extremely tightly to angiogenin, thereby abolishing both its biological and enzymatic activities (5). Since a vast amount of exceptionally detailed chemical information on RNase is available (reviewed in refs. 6 and 7) it should be possible to use it to advantage with angiogenin to (*i*) identify specific amino acids involved in its function and (*ii*) compare their reactivities with the corresponding residues in RNase. The results could both confirm the similarities between the two proteins and reveal interesting differences. We have therefore undertaken a systematic comparison of the characteristics of angiogenin and RNase A using the chemical modification approaches that have proved to be so helpful to the present understanding of the structure and function of RNase.

Numerous reagents selectively modify the active site residues His-12, Lys-41, or His-119 of RNase A; they include haloacetates (8–10) and their derivatives (11, 12), ferrate ion (13), 1-fluoro-2,4-dinitrobenzene (FDNB) (14), and 2-carboxy-4,6-dinitrochlorobenzene (15). In addition, phenylglyoxal inactivates RNase A by reacting with Arg-39 and Arg-85 (16). We find that the functional consequences of chemical modification of angiogenin are much like those with RNase A, suggesting that it too contains critical histidine, lysine, and arginine residues. However, the essential histidine and lysine groups in angiogenin appear to be less reactive than those in RNase A, whereas arginine modification is much faster. At least in part, these changes are accounted for by differences in the environments of these residues in the two proteins.

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MATERIALS AND METHODS

Angiogenin was isolated from human plasma as described in detail elsewhere (17). Concentrations of stock solutions of the protein were determined by amino acid analysis using the PicoTag method (18).

Camphorquinone-10-sulfonic acid, citraconic anhydride, and *p*-hydroxyphenylglyoxal were purchased from Pierce. Acetic anhydride, bromoacetic acid, phenylglyoxal, and sodium cyanoborohydride were products of Aldrich. Diethylpyrocarbonate, FDNB, poly(G), pyridoxal phosphate (PLP), tRNA (type X from yeast), and human serum albumin were obtained from Sigma. Calf liver rRNA (28S and 18S) was purchased from Pharmacia.

Chemical modifications were performed by standard procedures as described below using 14–30 μM angiogenin. Effects of chemical modification on enzymatic activity were assessed by two methods. First, activity toward 28S and 18S rRNA was examined using agarose gel electrophoresis followed by ethidium bromide staining as described (4). Activity of modified angiogenin was evaluated visually by comparing the extent of RNA degradation produced by 1.2 μg of this material in 30 min at 37°C with that observed using 0.06, 0.12, 0.24, 0.40, 0.60, 0.90, and 1.2 μg of unmodified angiogenin. More precise activity determinations were made by using a second assay, which measures formation of acid-soluble fragments from tRNA (see legend for Table 1).‡ In all but two instances, as noted, the effects of chemical modifications on ribonucleolytic activity were examined using both of the assays described. Results obtained by the two methods were in complete agreement. Appropriate controls in all cases demonstrated that the incubation conditions used do not influence angiogenin activity in the absence of modifying reagent.

The reduction of disulfide bonds in angiogenin and their subsequent re-formation was performed by a modification of the method of Anfinsen and Haber (19). Angiogenin was

Abbreviations: FDNB, 1-fluoro-2,4-dinitrobenzene; PLP, pyridoxal phosphate.

‡As noted previously (17), very high concentrations of angiogenin can produce a small amount of acid-soluble material from RNA under conditions generally used for measurement of RNase A activity. In this precipitation assay, the pH and ionic strength have been optimized to measure activity using somewhat lower angiogenin concentrations (0.2 μM).

incubated in 8 M urea containing 0.28 M 2-mercaptoethanol (pH 8.5) for 16 hr at room temperature under nitrogen. After gel filtration on Sephadex G-25 (fine) in 0.1 M acetic acid, this material was brought to pH 7.8 by addition of 1.2 vol of 0.5 M Hepes (pH 8.2) and incubated for 22 hr at room temperature.

C18 HPLC of phenylglyoxal-modified angiogenin was performed using the same system as for the unmodified protein (17) except that elution was achieved with a 60-min linear gradient from 30–70% solvent B.

Amino acid analyses of modified angiogenin were performed after desalting the samples by either C18 HPLC or gel filtration on G-25.

RESULTS

Histidine Modification. Treatment of angiogenin with 30 mM bromoacetate at pH 5.5 for 14 hr decreases its ribonucleolytic activity by 95% (Table 1). During this time, 1.5 of the 6 histidines are modified but no other changes in amino acid composition are evident. Inactivation is a first-order process with $t_{1/2}$ = 2.7 hr, 5-fold slower than that measured for bovine pancreatic RNase under identical conditions (Fig. 1).

Sodium phosphate (200 mM, pH 5.5) markedly decreases the rate of the bromoacetate inactivation so that 90% activity remains after 8 hr. In the absence of phosphate, incubation with bromoacetate at the same pH and at comparable ionic strength (50 mM sodium acetate, 150 mM NaCl) inactivates 60%.

Lysine Modification. A variety of lysine reagents also inactivate angiogenin (Table 1). Acylation with citraconic anhydride, acetic anhydride, or diethylpyrocarbonate abolishes activity almost completely. Acidification of citraconylated angiogenin (pH 3.5, 30°C, 4 hr) restores activity to 85%, consistent with inactivation by means of lysine acylation (20). Hydroxylamine (0.5 M, 1 hr, 27°C) does not appreciably reverse the effects of acetic anhydride and diethylpyrocarbonate; activity increases only to 8% and 2%, respectively. Hydroxylamine itself does not affect activity significantly. Thus, acetic anhydride and diethylpyrocarbonate likely inactivate by modifying lysine, since *O*-acyl tyrosine and *N*-acyl histidine are labile to hydroxylamine.

FDNB, formaldehyde plus sodium cyanoborohydride, or PLP plus sodium borohydride, all of which modify lysine, also cause significant loss of enzymatic activity (Table 1). In the absence of reducing agent, neither of the aldehydes inactivate; borohydride and cyanoborohydride alone also have little effect on activity. Amino acid analysis reveals that these reagents modify 3.8, 6.3, and 2.0 of the 7 lysine residues of angiogenin, respectively, under the conditions listed in Table 1. The rates of inactivation of angiogenin by FDNB and formaldehyde/$NaCNBH_3$ (Fig. 2) are ≈3 and 10 times slower, respectively, than those observed for RNase A under identical conditions. The extent of inactivation by PLP/$NaBH_4$ is somewhat less than that reported for RNase A (21).

Phosphate inhibits angiogenin and partially protects it from the effects of reductive methylation at pH 6. Thus, after treatment for 2 hr at the same ionic strength in the presence and absence of 0.2 M phosphate, activity decreases by 40% and 70%, respectively. Poly(G)[§] (2.5 mM), another angiogenin inhibitor, protects almost completely.

Arginine Modification. Incubation of angiogenin with the arginine reagents phenylglyoxal, *p*-hydroxyphenylglyoxal (22), and camphorquinone-10-sulfonic acid (23) also results in extensive loss of ribonucleolytic activity (Table 1). Treatment of bovine RNase A with phenylglyoxal and *p*-hydroxyphenylglyoxal (Fig. 3) under identical conditions inactivates at 4- and 5-fold slower rates, respectively. The effects of camphorquinone-10-sulfonic acid on angiogenin can be partially reversed by addition of 0.2 M *o*-phenylenediamine [pH 9.0, in 16% (vol/vol) ethanol (23)]. About 50% activity[¶] is restored after 20 hr at 37°C.

Phenylglyoxal-modified angiogenin elutes from a C18 HPLC column as a series of poorly resolved peaks, suggesting that several residues have been modified. This is confirmed by amino acid analysis of pooled fractions from these peaks, which reveals a loss of 6.7 of the original 13 arginine residues but no significant change in lysine content. The pooled material has 8% of the native enzymatic activity. Inactivation can be attributed to arginine rather than lysine modification, since any imines formed should decompose

Table 1. Effects of various reagents on the ribonucleolytic activity of angiogenin

Reagent	pH	Time, min	Activity,* $v/v_c \times 100$
None			100
Bromoacetate (30 mM)	5.5	840	5
Citraconic anhydride† (30 mM)	8.0	30	3
Diethylpyrocarbonate (2 mM)	7.5	10	1
Acetic anhydride (3 mM)	7.5	10	1
Fluorodinitrobenzene (50 mM)	8.0	60	20
Formaldehyde (2 mM) + sodium cyanoborohydride (10 mM)	6.0	90	18
Pyridoxal phosphate (0.5 mM) + sodium borohydride (2 mM)	6.0	30, 10	41
Phenylglyoxal (10 mM)	8.0	90	3
Hydroxyphenylglyoxal (10 mM)	8.0	90	1
Camphorquinone-10-sulfonic acid (5 mM)	9.0	780	15
2-Mercaptoethanol‡ (280 mM)	8.5	240	1
N-Acetylimidazole (20 mM)	7.5	90	84
Tetranitromethane (5 mM)	8.0	240	72
Carbodiimide§ (10 mM) + glycine ethyl ester (10 mM)	4.5	120	91
Me_2SO (100 mM)/HCl (1 M)	—	90	84

Angiogenin was isolated from human plasma using cation-exchange, phenyl, and C18 chromatography as detailed elsewhere (17). All reactions were at 27°C. The buffers used were 25 mM Mes (pH 4.5 and 6.0), 50 mM sodium acetate (pH 5.5), 25 or 50 mM Hepes (pH 7.5 and 8.0), and 25 mM Ches (pH 9.0). Me_2SO, dimethyl sulfoxide.

*Activities are listed as $v/v_c \times 100$ where v and v_c are reaction velocities in the presence and absence of modification reagent, respectively, and were determined using the tRNA assay in all cases except for the FDNB modification, where activity was evaluated using the agarose gel assay with 28S and 18S rRNA as substrate. For the tRNA assay, reaction mixtures containing 33 mM Hepes, pH 7.0, 33 mM NaCl, 0.6 mg of tRNA, and 30 μg of human serum albumin, in a vol of 300 μl were incubated at 37°C for 4 hr. The reaction was stopped by addition of 700 μl of ice-cold 3.4% perchloric acid, and after 10 min on ice the samples were centrifuged at 15,600 × *g* for 10 min at 4°C. The absorbance of the supernatant was measured at 260 nm in a 1-cm cuvette. Under these conditions, 1 μg of unmodified angiogenin produces an absorbance change of ≈0.8. Activity of modified angiogenin was quantitated by a standard curve, obtained using 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μg of unmodified angiogenin. Absorbance readings were corrected for any contribution of the modification reagents.

†Citraconic anhydride was added in three 10 mM aliquots. The reaction mixture was incubated for 10 min after each addition and was adjusted to pH 8 with sodium hydroxide prior to the second and third additions.

‡Incubations were in 8 M urea.

§1-Ethyl-3(3-dimethylaminopropyl)carbodiimide.

§This inhibition was measured using the tRNA assay.

¶Activity was determined using the rRNA assay.

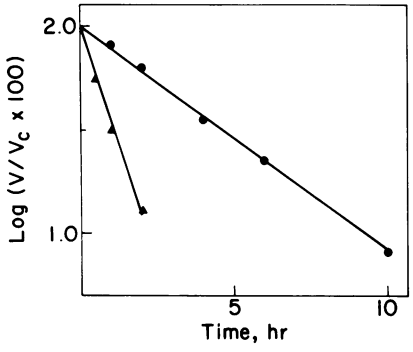

FIG. 1. Effects of bromoacetate on activity of angiogenin (●) and bovine pancreatic RNase A (▲). Reactions were performed with 30 mM bromoacetate in 50 mM sodium acetate (pH 5.5) at 27°C. Activities were measured with the tRNA assay described in the legend to Table 1.

under the conditions of C18 chromatography and more stable lysine adducts should decrease the lysine content measured by amino acid analysis.

Poly(G) (2.5 mM) affords virtually complete protection against inactivation by both phenylglyoxal and *p*-hydroxyphenylglyoxal (Fig. 3). Phosphate, however, does not protect. Thus, under conditions (20 mM *p*-hydroxyphenylglyoxal/50 mM Mes/0.2 M NaCl, pH 6.0, for 4 hr at 27°C) sufficient to cause 65% inactivation in the absence of phosphate, there is 74% inactivation when 0.2 M sodium phosphate replaces the Mes and NaCl.

Reduction of Cystines. Incubation of angiogenin with mercaptoethanol in 8 M urea for 4 hr decreases activity by >99% (Table 1). C18 HPLC of an aliquot of this material reveals a single peak eluting 16 min later than the native protein (Fig. 4). The remainder of the reduced angiogenin was desalted at pH 3 (see *Materials and Methods*) and after adjustment of the pH to 7.8, an aliquot was carboxymethylated with iodoacetate (25 mM, 45 min, 27°C), desalted on G-25, and subjected to amino acid analysis. The presence of 5.0 residues of carboxymethylcysteine indicates that all three disulfide bonds have been reduced. The remainder of the reduced angiogenin was incubated for 22 hr at room temperature and then chromatographed on the C18 column. Essentially all of the protein elutes as a single peak at a position identical to that of native angiogenin and is ≥67% active.

Additional Modifications. Tetranitromethane, *N*-acetylimidazole, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and 0.1 M dimethyl sulfoxide in 1 M HCl (24) do not significantly affect angiogenin activity (Table 1). Apparently,

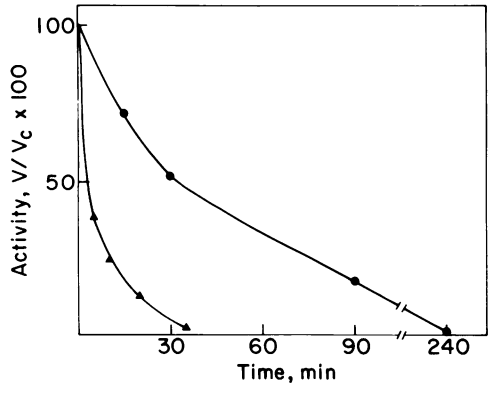

FIG. 2. Effects of reductive methylation on activity of angiogenin (●) and bovine RNase A (▲). Reactions were performed with 2 mM formaldehyde and 10 mM sodium cyanoborohydride in 25 mM Mes (pH 6.0) at 27°C. Activities were measured as described in Fig. 1.

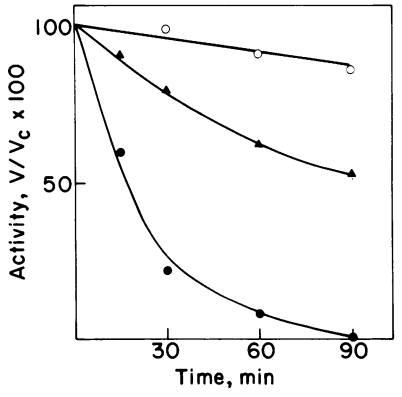

FIG. 3. Effects of *p*-hydroxyphenylglyoxal on activity of angiogenin (●, ○) and bovine RNase A (▲). Reactions were performed with 10 mM reagent in 25 mM Hepes (pH 8.0) at 27°C. Angiogenin incubations were in the presence (○) or absence (●) of 2.5 mM poly(G).

angiogenin lacks reactive tyrosine, cysteine, carboxyl, or methionine groups critical to its activity.

DISCUSSION

The recently recognized sequence homology between angiogenin and RNase provides a compelling reason to directly compare the two proteins using the extensive body of chemical and structural information available on RNase. This information has greatly benefited the understanding of the structure–function relationships and mechanism of action of RNase. It acquires additional importance for its capacity to indicate approaches for gaining a comparable understanding of angiogenin. Thus, a preliminary three-dimensional structure of angiogenin has been computed by minimization of its conformational energy based on the assumption that its backbone structure is similar to that of RNase (25). The results to date argue for conservation of the overall structural motif of RNase in the angiogenin molecule and are entirely consistent with its being a ribonucleolytic enzyme.

A more specific examination of the critical nature of individual amino acids in angiogenin is possible by selective chemical modifications, since this is the most direct means for identifying residues involved in catalysis. Results from past work of this sort on RNase A are particularly abundant and the conclusions have been verified by a variety of

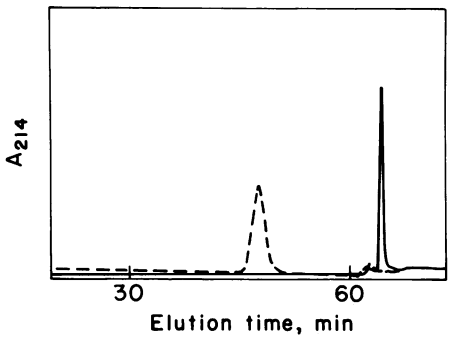

FIG. 4. Chromatography of native (broken line) and reduced (solid line) angiogenin on a C18 HPLC column. Reduction was performed as described in the text. Reduced angiogenin, in 0.28 M 2-mercaptoethanol/8 M urea, pH 8.5, was diluted 1:26 into 0.1% trifluoroacetic acid (solvent A) prior to injection. Elution was achieved with a linear gradient of 28–40% solvent B in 54 min followed by a linear gradient of 40–70% solvent B in 20 min at 1 ml/min, where solvent B is 2-propanol/acetonitrile/water, 3:2:2, in 0.08% trifluoroacetic acid.

physicochemical approaches but particularly crystallographic analysis. Therefore it is possible to use these studies as the basis for the examination of a number of important questions regarding angiogenin. Does it employ the same (i.e., corresponding) residues for enzymatic activity as RNase A? Is its lack of pancreatic RNase-like activity paralleled by a concomitant decrease in chemical reactivity of the active site residues that the two proteins share? Are there additional residues or residue classes, not critical for RNase A function but important for angiogenin's characteristic angiogenic and/or ribonucleolytic activities? Do the two activities of angiogenin depend on the same or different residues?

Among the multitude of well-characterized modification reactions used previously with RNase A, carboxymethylation of His-12 and His-119 with bromo- or iodoacetate at acidic pH (8, 9) and alkylation of Lys-41 by FDNB (14) or with bromoacetate at alkaline pH (10) are considered classical. All three of these residues have counterparts in the sequence of angiogenin, as His-13, Lys-40, and His-114. It is not surprising, therefore, that angiogenin is also inactivated by these reagents. As with RNase A the reaction with bromoacetate at pH 5.5 is specific, but the rate of inactivation is 5-fold slower (Fig. 1). This decreased rate may derive in part from the presence of leucine at the position corresponding to Phe-120 of RNase A. Lin *et al.* (26) have reported a similarly lower inactivation rate with a semisynthetic RNase derivative containing this substitution.

The inactivation of angiogenin by FDNB is also considerably slower than with RNase A. In this case, however, the angiogenin reaction clearly lacks the specificity observed with the pancreatic enzyme. RNase A loses 75% of its activity on modification of only 1.4 of 10 lysines (14), primarily Lys-41 and, to lesser extents, Lys-7 and the α-amino group of Lys-1. These latter two reaction sites do not exist in angiogenin since its N terminus is blocked and histidine occupies the position corresponding to Lys-7. Nevertheless, 3.8 of its 7 lysines are modified under conditions where its ribonucleolytic activity is decreased by 80%.

Reductive methylation of angiogenin reveals a similar lack of selectivity together with a decreased rate of inactivation compared to that with RNase A. It is inactivated $\approx$10-fold more slowly than RNase A, and to achieve 82% inactivation 90% of the lysines have to be modified. Reductive pyridoxylation decreases RNase A activity by 80%, modifying Lys-7 and Lys-41 in a 2:3 ratio and in a mutually exclusive manner (21). Despite the absence of the first of these sites in angiogenin, treatment with PLP/$NaBH_4$ decreases ribonucleolytic activity by only 59% while modifying 2.0 of the 7 lysines.

Although reductive methylation of angiogenin is accompanied by the modification of multiple lysines, the resultant loss of activity is probably due to modification of a specific residue (or residues) rather than to a general effect on protein conformation. Methylation does not alter the pK_a values of lysines significantly (27) nor would it be expected to cause extensive steric perturbations. Nevertheless, it results in almost complete inactivation, which can be prevented by phosphate, suggesting that the effect is due to reaction at the active site. Phosphate is a competitive inhibitor of RNase A and protects it against inactivation by numerous reagents (9, 14). Phosphate also inhibits the enzymatic activity of angiogenin,[§] although the mode of this inhibition has yet to be determined.

On the basis of the angiogenin RNase A sequence homology, it seems likely that Lys-40 of angiogenin is a functionally critical residue. The high reactivity of the corresponding RNase A residue—Lys-41—with modification reagents is probably due in part to its abnormally low pK_a value of $\approx$8.8 (28). This pK_a is thought to reflect the presence of neighboring positive charges, such as Arg-39 (29) and Lys-7 (30). It has been suggested (see ref. 31) that the relatively low reaction rate of FDNB with Lys-41 in rat pancreatic RNase is a consequence of the substitution of Arg-39 by serine. Depending on the alignment chosen, in angiogenin there is either a deletion or a proline at the position corresponding to Arg-39, and Lys-7 is replaced by a histidine. Thus, if Lys-40 of angiogenin is indeed critical for activity, these substitutions may account for its decreased chemical reactivity compared with Lys-41 of RNase A.

Treatment with arginine reagents also inactivates angiogenin, indeed much faster than RNase A (Fig. 3). Inactivation is also nonspecific, raising questions regarding general conformational causes. However, the complete protection conferred by the inhibitor poly(G) suggests that a critical residue may be involved.

Inactivation of RNase A by arginine reagents has been thought to involve arginines at positions 39 and 85 (16). Angiogenin has 13 arginines but none of them corresponds to either of these positions. Hence, a different arginine must be involved in loss of activity. Based on homology considerations, those at positions 5, 21, 24, 33, 122, and 123 would seem unlikely candidates, but those at positions 66 and 70 are in or near the region corresponding to that where purines bind in RNase A.

Chemically reactive, functional methionine, tyrosine, aspartic acid, or glutamic acid residues have not been observed in angiogenin. Its one methionine corresponds to Met-30 of RNase A, which can be alkylated without diminishing activity (32). Similarly, modification of tyrosines in RNase A with tetranitromethane also does not affect activity (33). Of the 11 carboxyl groups in angiogenin, Asp-15, Glu-108, and Asp-116 correspond to residues in RNase A that are thought to be involved in important hydrogen bonds. Modification of the second of these residues may decrease RNase activity by $\approx$40%, while the other two are relatively resistant to derivatization (34). Similar reaction conditions, however, do not affect the enzymatic activity of angiogenin.

Chemical modification of angiogenin can address the relationship between its angiogenic and ribonucleolytic activities. As reported previously (4), treatment of angiogenin with bromoacetate at pH 5.5 markedly decreases both activities. The present results indicate that these effects can probably be attributed to modification of the same amino acid residue(s), since only 1.5 histidines have been derivatized. Concerning the role of enzymatically critical lysines and arginines in the biological activity of angiogenin, the rigorous examination of this issue must await the development of more selective modifications.

In this regard, oligonucleotide-directed mutagenesis, yet to be performed on either angiogenin or RNase, should provide complementary information and extend this examination of their structure–function relationships. Indeed, the present results should serve as a solid foundation for future comparisons of the two proteins by this technique. For the most part, the chemical modifications have borne out homology-based expectations indicating the presence of critical histidine and lysine residues. In addition, they suggest that arginines, different from those implicated in the action of RNase, play an important role in the enzymatic activity of angiogenin.

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