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Enzymatically Active Angiogenin/Ribonuclease A Hybrids Formed by Peptide Interchange†

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ABSTRACT: The primary structures of the blood vessel inducing protein human angiogenin and human pancreatic ribonuclease (RNase) are 35% identical. Angiogenin catalyzes the limited cleavage of ribosomal RNA (18 and 28 S), yielding a characteristic pattern of polynucleotide products, but shows no significant activity toward conventional pancreatic RNase substrates [Shapiro, R., Riordan, J. F., & Vallee, B. L. (1986) *Biochemistry 25*, 3527–3532]. Angiogenin/RNase hybrid enzymes—wherein particular regions of primary structure in RNase are replaced by the corresponding segments of angiogenin—serve to explore the structural features underlying angiogenin's characteristic activities. Herein we show that synthetic angiogenin peptides, Ang(1–21) and Ang(108–123), form noncovalent complexes with inactive fragments of bovine RNase A—RNase(21–124) (i.e., S-protein) and RNase(1–118), respectively—with regeneration of activity toward conventional RNase substrates. Maximal activities for the Ang(1–21)/S-protein complex (K_d = 1.0 μM) are 52%, 45%, and 15% toward cytidine cyclic 2′,3′-phosphate, cytidylyl(3′→5′)adenosine, and yeast RNA, respectively. In contrast, activities of the RNase(1–118)/Ang(108–123) hybrid (K_d = 25 μM) are 1–2 orders of magnitude lower toward cyclic nucleotides and dinucleoside phosphates. However, substitution of phenylalanine for Leu-115 in Ang(108–123) increases activity up to 100-fold. Both His-13 and His-114 in the angiogenin peptides are required for activity since their substitution by alanine yields inactive complexes. Importantly, the pattern of polynucleotide products formed during cleavage of ribosomal RNA by the Ang(1–21)/S-protein hybrid shows a striking resemblance to that formed by angiogenin, demonstrating that the hybrid retains features of both angiogenin and RNase A. In contrast, neither RNase(1–118)/Ang(108–123) nor S-peptide/S-protein complexes produce this cleavage pattern. Thus, the data point to an important role for the N-terminal region of angiogenin in conferring substrate selectivity.

Human angiogenin is a blood vessel inducing protein whose primary structure is 35% identical with that of human pancreatic ribonuclease (RNase)[1] (Fett et al., 1985; Strydom et al., 1985; Kurachi et al., 1985). Preliminary energy minimization and molecular modeling studies indicate that the polypeptide backbones of the two proteins are readily superimposable within the regions of their α-helical and β-sheet structures (Palmer et al., 1986). The three active site residues identified for RNase—His-12, Lys-41, and His-119—have direct counterparts in angiogenin, and indeed, angiogenin has been found to exhibit ribonucleolytic activity. This activity differs markedly, however, from that of pancreatic RNase: it

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[1] Abbreviations: RNase, pancreatic ribonuclease; RNase A, bovine pancreatic ribonuclease A; C>p, cytidine cyclic 2′,3′-phosphate; U>p, uridine cyclic 2′,3′-phosphate; UpN and CpN, uridine and cytidine 3′,5′-dinucleoside phosphates where N represents adenine or guanine; K_d, apparent dissociation constant for the peptide/protein complex; Mes, 2-(*N*-morpholino)ethanesulfonic acid; C18, octadecylsilane; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)-aminomethane; TFA, trifluoroacetic acid; Boc, *tert*-butyloxycarbonyl; Tos, *p*-toluenesulfonyl; Bzl, benzyl; Br-Z, (2-bromobenzyl)oxycarbonyl; Cl-Z, (2-chlorobenzyl)oxycarbonyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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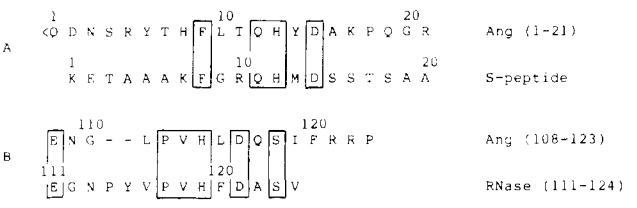

FIGURE 1: (A) Amino acid sequence of Ang(1–21) and its alignment with S-peptide. (B) Amino acid sequence of Ang(108–123) and its alignment with RNase(111–124). The numbering systems employed are those of angiogenin (Strydom et al., 1985) and bovine RNase A (Richards & Wyckoff, 1981), respectively. Identical residues are boxed. <Q designates pyroglutamic acid.

results in limited cleavage of 18S and 28S ribosomal RNA but is not evident with more conventional RNase substrates such as C>p or homonucleotide polymers (Shapiro et al., 1986a).

The molecular events leading to the induction of blood vessel formation by angiogenin or any other angiogenic agent are currently unknown. However, the recognition of angiogenin's ribonucleolytic activity raises the possibility that its action on RNA may be instrumental in this process. Elucidation of the molecular basis of this action, therefore, could be extremely useful in the understanding of angiogenesis and might well lead to the discovery of new approaches for controlling this process in vivo. The angiogenin/RNase homology suggests a variety of means for carrying out such studies.

The present report describes noncovalent angiogenin/RNase hybrid enzymes and demonstrates their utility as experimental tools for exploring the contributions of various segments of the primary structure of angiogenin to its function. The initial series of hybrid enzymes was designed on the basis of the ability of N- and C-terminal peptides of RNase A—S-peptide[2] and RNase(111–124), respectively—to combine noncovalently with the complementary inactive "core" fragments, S-protein and RNase(1–118), to regenerate catalytic activity (Richards, 1958; Richards & Vithayathil, 1959; Lin, 1970; Lin et al., 1970). Such studies have been extremely valuable in elucidating the structure and mechanism of RNase and have revealed how critical the N- and C-terminal portions of the protein are to its function [for reviews see Richards and Wyckoff (1971) and Blackburn and Moore (1982)]. Herein, we show that the synthetic angiogenin peptides (Figure 1) corresponding to the S-peptide and RNase(111–124) can also combine with the appropriate core RNase fragments to regenerate catalytic activity toward a variety of substrates. The results afford insight into the molecular basis for the differences in the nucleolytic activities of angiogenin and RNase.

Materials and Methods

Materials. Dinucleoside phosphates, cytidine cyclic 2′,3′-phosphate (C>p), uridine cyclic 2′,3′-phosphate (U>p), bovine RNase S-protein, bovine RNase S-peptide, pepsin, and human serum albumin were obtained from Sigma Chemical Co. Yeast RNA was purchased from Calbiochem-Behring, calf liver rRNA (18 and 28 S) was from Pharmacia, bovine RNase A was from Cooper Biomedical, and human angiogenin was isolated from normal human plasma as described (Shapiro et al., 1987). Bovine carboxypeptidase A (Sigma) was purified by affinity chromatography on 5-aminocaproyl-4-aminobenzylsuccinyl-Sepharose resin to remove contaminating serine proteases (Bicknell et al., 1985).

The following side-chain-protected α-Boc L amino acids were obtained from Peninsula Laboratories: Arg(Tos), Asp(Bzl), Glu(Bzl), Ser(Bzl), Thr(Bzl), Lys(Cl-Z), His(Tos), and Tyr(Br-Z). Chloromethylstyrene–divinylbenzene resin (1% cross-linked, 0.7 mmol of Cl^-/g, 200–400 mesh) was purchased from Lab Systems, Inc. Amino acids, *N*,*N*′-dicyclohexylcarbodiimide (DCC), L-pyroglutamic acid, and Boc anhydride were obtained from Fluka Chemical Corp. [^{3}H]-L-Phe (20 Ci/mM) and [2,3,4,5-^{3}H]-L-Pro (100 Ci/mM) were from ICN Pharmaceuticals. Tritiated Boc-Phe and Boc-Pro were prepared from the reaction of Boc anhydride with the tritiated amino acid. Trifluoroacetic acid (Halocarbons) and dichloromethane (E. M. Science) were distilled prior to use. Triethylamine and dimethylformamide were Sequanal grade (Pierce Chemical Co.).

RNase S-peptide was separated from contaminating RNase A and S-protein by HPLC as described below for synthetic angiogenin peptides.[3]

RNase S-protein was separated from contaminating RNase A (~2%) and S-peptide by chromatography on agarose–hexane–uridine 5′-triphosphate (P-L Biochemicals). S-Protein (2.5 mg) in 25 mM sodium acetate, pH 5.5 (starting buffer), was applied to resin (0.6 × 2 cm) equilibrated with starting buffer. The column was washed with 10 mL of starting buffer (flow rate 5 mL/h) and the S-protein then eluted with this buffer containing 0.15 M NaCl. Preparations of S-protein contained less than 0.03% RNase A (CpA as substrate).

Peptide Synthesis. Synthetic angiogenin peptides were prepared by standard solid-phase methodologies (Barany & Merrifield, 1980) using the Boc/Bzl strategy for weak acid removal of α-amino protecting groups and strong acid removal of side-chain protecting groups. The first Boc amino acid was anchored to the support by refluxing chloromethylstyrene–divinylbenzene resin (1 equiv of reactive site) with 2 equiv of Boc amino acid and 1.8 equiv of triethylamine in absolute ethanol for 24–65 h at 80 °C. Subsequent Boc amino acids (except Boc-Gln and Boc-Asn) were added by repeated cycles of deprotection with 50% (v/v) TFA in dichloromethane, deprotonation with 5% (v/v) triethylamine in dichloromethane, and coupling with DCC in dichloromethane. Boc amino acid and DCC were added in 5-fold molar excess to available amino termini. At the positions of proline and phenylalanine, tritiated Boc amino acids were used. Activated esters of Boc-Gln and Boc-Asn were prepared immediately prior to use from hydroxybenzotriazole and DCC (König & Geiger, 1972). The extent of peptide coupling was judged by performing a modified Kaiser test (Sarin et al., 1981) at each step of the reaction, and additional coupling reactions were performed as necessary. Side chains were deprotected and peptides cleaved from the resin by using HF (10 mL/g of resin) at 0 °C for 1 h followed by 0–20 °C for 1 h, in the presence of anisole (9:1 HF:anisole). Crude peptides were washed with ethyl acetate, extracted with 1% acetic acid, and lyophilized twice. Peptides were purified

[2] The term S-peptide has been commonly used to designate RNase (1–20), the N-terminal component of RNase S derived from subtilisin cleavage of RNase A (Richards & Vithayathil, 1959). S-Protein refers to the protein component of RNase S, RNase(21–124). The nomenclature employed to designate angiogenin peptides and other RNase fragments is based on an adaptation of the rules of IUPAC–IUB commission on Biochemical Nomenclature (1967). Thus, "Ang(X–Y)" refers to an angiogenin peptide whose N- and C-terminal amino acids, respectively, are denoted by X and Y, corresponding to their positions in the primary structure of angiogenin (Strydom et al., 1985). Analogous abbreviations are used for RNase fragments 1–118 and 111–124.

[3] S-peptide obtained from Sigma Chemical Co. has been shown previously to be RNase(1–19) rather than the 20-residue fragment RNase(1–20) (Silverman et al., 1972; Kuwajima & Baldwin, 1983). The amino acid composition of our HPLC-purified S-peptide preparation indicates that it is RNase(1–19).

by preparative reversed-phase HPLC using linear gradients of 0.1% TFA/acetonitrile in water and/or 0.1% TFA/methanol in water on a Waters μBondapak C18 column (19 mm × 150 mm) at a flow rate of 6 mL/min. Amino acid compositions of purified peptides were within 5% of that expected.

Preparation of RNase(1–118). RNase(1–118) was prepared from RNase A by treatment with pepsin followed by carboxypeptidase A according to a modification of the procedure described by Lin (1970). RNase A (10 mg/mL in 0.1% HCl, pH 2) was incubated with 70 nM pepsin for 20 min at 37 °C. The reaction was quenched by addition of 10 volumes of 100 mM sodium phosphate, pH 6.0, and applied to a Mono-S cation-exchange column (50 mm × 5 mm, Pharmacia). RNase(1–120) was eluted with a 60-min linear gradient from 0 to 200 mM NaCl in 100 mM sodium phosphate, pH 6.0, at a flow rate of 0.8 mL/min. Fractions containing RNase(1–120) were desalted by reversed-phase HPLC on a Synchropak RP-P (C18) column (250 mm × 4.1 mm, Synchrom Inc.) using linear gradients of 2-propanol, acetonitrile, and water containing 0.1% TFA. Peak fractions were lyophilized, dissolved in 50 mM Hepes, pH 7.5, containing 0.1 M NaCl to give a concentration of 440 μM, and then digested with carboxypeptidase A (50 nM; 47 °C, 20 min). The reaction mixture was fractionated by cation-exchange HPLC as described above for RNase(1–120). Fractions containing RNase(1–118) were dialyzed against water. RNase(1–118) preparations showed less than 0.01% contaminating RNase A (C>p as substrate).

Protein Assays. The concentrations of all peptides and proteins were determined by amino acid analysis (Picotag, Waters Associates), except for that of RNase A which was based on absorbance at 280 nm by using $\epsilon_{280} = 9800\ M^{-1}\ cm^{-1}$ (Sela & Anfinsen, 1957).

Enzymatic Assays. Cleavage of yeast RNA was measured spectrophotometrically at 300 nm (Kunitz, 1946). Premixed peptide and protein components were added to yeast RNA (1 mg/mL) in 100 mM Tris, pH 7.5, and 100 mM NaCl at 25 °C, and the initial decrease in absorbance was measured continuously on either a Cary Model 219 or a Gilford Model 250 spectrophotometer.

In most cases, it was possible to determine activities toward dinucleoside phosphates, C>p, and U>p according to the spectrophotometric methods of Witzel and Barnard (1962), Crook et al. (1960), and Richards (1955), respectively. Peptide and protein components were premixed in 25 mM Mes, pH 6.0, and 100 mM NaCl, and an aliquot of substrate was added to give final concentrations of 40–100 μM and 0.5 mM for dinucleoside phosphates and cyclic nucleotides, respectively. Absorbance changes were monitored continuously at the appropriate wavelength (280–290 nm) on a Cary Model 219 spectrophotometer at 25 °C.

With some of the hybrid enzymes examined, the low level of activity necessitated the use of a more sensitive assay method based on HPLC quantitation of products and reactants (Shapiro et al., 1986a,b). Incubations were performed as above, unless noted otherwise, and at various times an aliquot (10–15 μL) of the reaction mixture was applied to a Waters Radial-PAK C18 column (type 8NVC 185, 5-μm particle size, 100 mm × 8 mm) equilibrated with 100 mM potassium phosphate, pH 7.0. For C>p and U>p, elution was achieved under isocratic conditions. The absorbance was monitored at 254 nm with a Waters Model 440 detector in combination with a Waters Model 740 integrator, and the product and reactant peak areas were used to calculate activities.

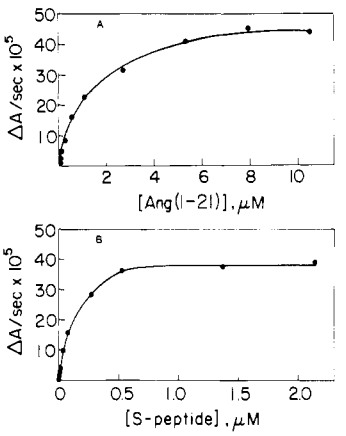

FIGURE 2: Activity of mixtures of (A) Ang(1–21) or (B) S-peptide with S-protein [2.0 nM for (A) and 1.2 nM for (B)] toward CpA. Peptide and protein components were premixed in 25 mM Mes, pH 6.0, and 0.1 M NaCl at 25 °C and reactions initiated by addition of CpA (final concentration 90 μM). The initial velocity was measured continuously at 286 nm. Each data point indicates the average of measurements performed in duplicate or triplicate. Apparent dissociation constants were calculated by the method of Levit and Berger (1976).

Activity toward calf liver rRNA (18 and 28 S) was assessed by agarose gel electrophoresis under denaturing conditions. rRNA (10–15 μg) was added to either angiogenin or premixed peptide and protein components in 30 mM Hepes, pH 7.5, and 30 mM NaCl in a total volume of 13 μL and the reaction mixture incubated at 37 °C. Reactions were quenched and the products examined on a 1.1% agarose gel containing 6% formaldehyde as described (Shapiro et al., 1986a). Bromophenol blue was used as a tracking dye. RNA was visualized with UV light after staining with ethidium bromide.

All peptide and protein dilutions were made in 0.01% human serum albumin in order to minimize losses due to adsorption.

RESULTS

Activity of N-Terminal Angiogenin Peptide/S-Protein Hybrid Enzymes. The hybrid enzyme prepared by addition of Ang(1–21)[2] to S-protein efficiently cleaves a variety of conventional RNase substrates, including dinucleoside phosphates and cyclic nucleoside monophosphates. When CpA is the substrate, the activity depends hyperbolically on peptide concentration; maximal activity is achieved with 8 μM peptide (Figure 2A). The kinetically determined peptide/protein dissociation constant (K_d) of 1.0 μM is within an order of magnitude of that determined for the interaction of S-peptide with S-protein, 0.11 μM (Figure 2B), while the maximal activities of the two complexes toward CpA differ by only 2-fold (Table I).

The k_{cat}/K_m values for cleavage of several dinucleoside phosphate substrates by the Ang(1–21)/S-protein hybrid complex and by RNase A are compared in Table I. The values for the hybrid enzyme range from 45% of that of RNase A with CpA to 7% with UpG. Slightly higher relative activities are obtained when the comparison is made with the combination of S-peptide and S-protein instead of RNase A. The hybrid enzyme also cleaves yeast RNA with 15% of the activity exhibited by RNase A (Table I). Thus, the Ang(1–21)/S-protein complex is active in the transphosphorylation step of RNA cleavage.

The hydrolytic activity of the Ang(1–21)/S-protein complex was examined with nucleoside 2′,3′-cyclic monophosphates. Compared with RNase A, the hybrid enzyme is 52% and 31% active toward C>p and U>p, respectively (Table I). Hence,

Table I: Activities of RNase A and Mixtures of S-Protein and either Ang(1–21), Ang(6–21), or S-Peptide[a]

substrate	RNase A k_{cat}/K_m[b]	Ang(1–21) k_{cat}/K_m[b]	Ang(1–21) %[c]	Ang(6–21) k_{cat}/K_m[b]	Ang(6–21) %[c]	S-peptide k_{cat}/K_m[b]	S-peptide %[c]
CpA	60.0	27.0	45	2.6	4.8	60.0	100
UpA	41.0	6.7	16	0.89	2.2	17.0	42
CpG	5.1	0.59	11	0.14	2.8	3.8	73
UpG	1.8	0.12	7	0.04	1.9	1.2	65
yeast RNA[d]			15[e]		5.9[f]		100
C>p[d]			52		7.7		95
U>p[d]			31				

[a] Assays were performed spectrophotometrically as described under Materials and Methods, employing a premixed peptide component of either 8 μM Ang(1–21), 100 μM Ang(6–21), or 8 μM S-peptide and S-protein at 1.2 nM–2.0 μM depending on the substrate being examined. [b] Units of k_{cat}/K_m are $M^{-1}\ s^{-1} \times 10^{-5}$. Under the conditions employed for the dinucleoside phosphates ([S] = 40 μM $\ll K_m$), reaction velocities were first order in substrate, allowing k_{cat}/K_m values to be calculated from the observed first-order rate constant for substrate cleavage according to $k_{obsd} = [E_T]k_{cat}/K_m$. [c] Percent activities are relative to rates measured with RNase A (1–65 nM). [d] Activities are based on initial velocities. [e] Identical activities were found with 4 μM Ang(1–21). [f] Peptide concentration was 10 μM and was saturating.

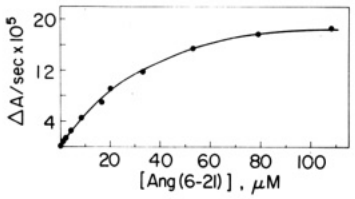

FIGURE 3: Activity of mixtures of S-protein as a function of increasing concentration of Ang(6–21) with CpG as substrate. Assays were performed as described for Figure 2 with 355 nM S-protein and 90 μM CpG.

it is also effective in the cyclic nucleotide hydrolysis step of RNA cleavage.

Ang(6–21), a derivative of Ang(1–21) that lacks the first five amino acids at the N-terminus, also forms a catalytically active complex with the S-protein. The dependence of activity on peptide concentration (Figure 3) shows that the K_d for the complex is 25 μM (CpG as substrate). Compared with RNase A, maximal activities range from 1.9% to 7.7% (Table I) and are 2.5–9-fold lower than the activities observed for the Ang(1–21)/S-protein hybrid.

Activity of RNase(1–118)/C-Terminal Angiogenin Peptide Hybrid Enzymes. The activities of mixtures of RNase(1–118) and either Ang(108–123) or Ang(108–122) toward several substrates are given in Table II. With yeast RNA, the activities of these hybrid enzymes are 11% of that of RNase A. The activities toward dinucleoside phosphates and cyclic nucleoside monophosphates are from 1 to 2 orders of magnitude lower. The K_d value for the RNase(1–118)/Ang(108–123) complex is 25 μM when measured with CpA as substrate and 7 μM when yeast RNA is the substrate. The activities found with Ang(108–123) and Ang(108–122) do not differ significantly (Table II), demonstrating that the C-terminal proline residue is not critical to function.

Previously, it was shown that substitution of Phe-120 in RNase(111–124) by leucine results in a 7-fold decrease in the activity of its RNase(1–118) complex toward C>p (Lin et al., 1972). Since angiogenin contains a leucine at the corresponding position (Figure 1), we synthesized the peptide [Phe[115]]Ang(108–123) in order to determine what effect this substitution has on activity. This replacement results in a dramatic increase in activity toward cyclic nucleotide and dinucleoside phosphate substrates of up to 100-fold (Table II). However, this substitution increases activity toward yeast RNA only slightly (Table II). The K_d for the peptide/protein complex, 50 μM, increases by 2-fold.

Table II: Activities of Mixtures of RNase(1–118) and either Ang(108–123), Ang(108–122), or [Phe[115]]Ang(108–123)[a]

substrate	Ang(108–123)[b] k_{cat}/K_m[c]	Ang(108–123)[b] %	Ang(108–122)[b] k_{cat}/K_m[c]	Ang(108–122)[b] %	[Phe[115]]Ang-(108–123)[b] k_{cat}/K_m[c]	[Phe[115]]Ang-(108–123)[b] %
CpA	0.3	0.5	0.25	0.4	11.4	19.2
UpA	0.04	0.1			4.4	10.2
CpG	0.02	0.4			0.29	5.6
UpG	ND[d]	<0.01			0.11	6.1
yeast RNA[e]		11		11		14
C>p		0.8[f]		0.8[g]		17[h]
U>p		0.3[f]		0.2[g]		10[h]

[a] Assays were performed spectrophotometrically as described under Materials and Methods with 3 nM–1 μM RNase(1–118) unless noted otherwise. Percent activities are relative to rates measured with RNase A (see Table I). [b] Peptide concentration was 110–230 μM unless otherwise noted. [c] Units of k_{cat}/K_m are $M^{-1}\ s^{-1} \times 10^{-5}$. Values were determined as described in Table I. [d] ND, not detectable; $k_{cat}/K_m < 10\ M^{-1}\ s^{-1}$. [e] Peptide concentrations were Ang(108–123) = 75 μM, Ang-(108–122) = 43 μM, and [Phe[115]]Ang(108–123) = 40 μM and were saturating. [f] Determined by HPLC (see Materials and Methods) with 0.53–1.1 μM RNase(1–118) and 420 μM Ang(108–123). [g] Determined by HPLC with 0.55–1.1 μM RNase(1–118) and 290 μM Ang(108–122). [h] Conditions were 618 μM [Phe[115]]Ang(108–123) with 0.2–0.44 μM RNase(1–118).

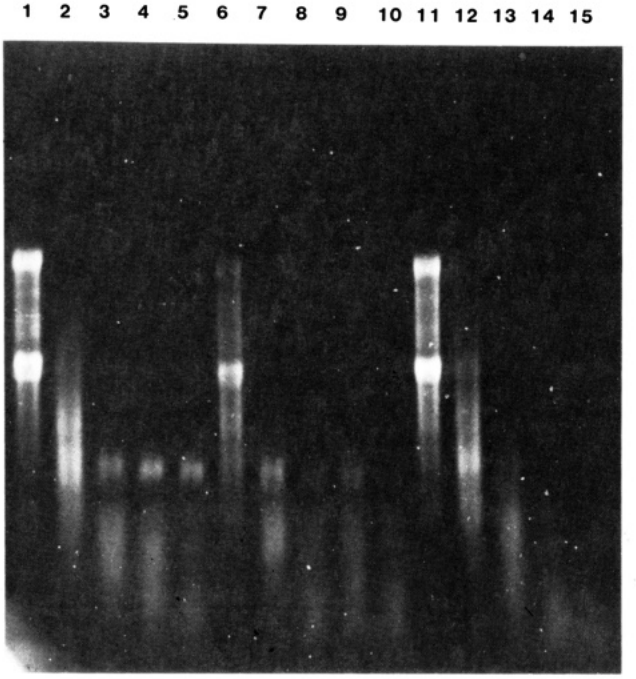

FIGURE 4: Time course for degradation of calf liver 18S and 28S rRNA by angiogenin, Ang(1–21)/S-protein, and S-peptide/S-protein. RNA was incubated with either peptide and S-protein components or angiogenin as described under Materials and Methods. Reactions were terminated by addition of a 15:5:3 mixture (v/v) of deionized formamide, 37% formaldehyde, and 200 mM 3-(*N*-morpholino)-propanesulfonic acid, pH 7.0, containing 50 mM sodium acetate and 10 mM EDTA. Samples were run on a 1.1% agarose gel under denaturing conditions. RNA was visualized with ethidium bromide (0.5 μg/mL). (Lane 1) Control RNA incubated with 1.5 nM S-protein for 120 min; (lanes 2–5) samples containing 1 μM angiogenin incubated for 3, 30, 60, and 120 min, respectively; (lanes 6–10) samples containing 10 μM Ang(1–21) and 1.5 nM S-protein incubated for 3, 10, 30, 60, and 120 min, respectively; (lanes 11–15) samples containing 10 μM S-peptide and 1.5 nM S-protein incubated for 3, 10, 30, 60, and 120 min, respectively.

Activities of Angiogenin/RNase Hybrids toward rRNA. Treatment of 18S and 28S rRNA with angiogenin results in the formation of a characteristic pattern of polynucleotide

products ranging from 100 to 500 nucleotides in length, as assessed by agarose gel electrophoresis (Shapiro et al., 1986a). In contrast, RNase A degrades both of these RNA species to much smaller oligonucleotides that migrate well ahead of the dye front. Therefore, the activities of the angiogenin/RNase hybrid enzymes were assessed with rRNA in order to allow comparison to angiogenin as well as RNase.

Figure 4 (lanes 2–4) shows the time-dependent production of polynucleotide products observed upon incubation of rRNA (lane 1) with 1 μM angiogenin. The distribution of the two major product bands does not change substantially even after extended incubation (lane 5). The combination of 10 μM Ang(1–21) and 1.5 nM S-protein also degrades both RNA species (Figure 4, lanes 6–10). A digest pattern strikingly similar to that obtained with angiogenin is evident after a 5-min incubation period (lane 7) and is maintained over the next 60-min period (lanes 8–9). Even after 120 min, these polynucleotide products are not substantially degraded (lane 10). When Ang(6–21) is the peptide component, the resultant hybrid enzyme generates a pattern of products that is virtually indistinguishable from that observed with Ang(1–21).

On the other hand, the cleavage pattern found with RNase S, i.e., the combination of 10 μM S-peptide and 1.5 nM S-protein (Figure 4, lanes 11–15), is closely similar to that observed with RNase A (not shown) and differs dramatically from that found with the Ang(1–21)/S-protein hybrid. RNase S degrades rRNA readily over the 120-min reaction period without any apparent accumulation of large intermediate polynucleotide products.

Additional assays were performed with 7-fold higher concentrations of S-protein in order to determine whether the Ang(1–21)/S-protein hybrid could further degrade the polynucleotide products shown in Figure 4 (lane 10). Under these conditions, bands similar to those characteristic of the action of angiogenin on rRNA can be seen after 3 min, but over a period of 120 min these initial products are largely broken down to species that migrate ahead of the dye front (data not shown). It should be noted that with angiogenin these products persist for much longer periods of time (Shapiro et al., 1986a).

The action of mixtures of RNase(1–118) (1.5 nM) and either Ang(108–123) or [Phe^{115}]Ang(108–123) (50 μM) on rRNA was also assessed. In both cases, the electrophoretic pattern of products resembles that of RNase A (or RNase S). Cleavage of both rRNA species (Figure 5, lane 1) occurs readily over a 60-min period yielding low molecular weight products that migrate well ahead of the dye front (Figure 5, lanes 2–6 and 7–11). There is no apparent accumulation of polynucleotide species as found with the Ang(1–21)/S-protein hybrid enzyme.

Requirement of His-13 and His-114 for Catalysis. On the basis of the homology with RNases, His-13 in Ang(1–21) and His-114 in Ang(108–123) should be required for catalysis. This has been confirmed by replacing these residues by alanine. A complex of [Ala^{13}]Ang(1–21) (60 μM) and S-protein (25–40 nM) exhibits no activity toward CpA or C>p under conditions where as little as 0.03% activity could have been detected. The combination of RNase(1–118) (140 nM) and [Ala^{114}]Ang(108–123) (80 μM) also shows no activity toward CpA. Competition assays (Hofmann et al., 1971) using CpA as substrate reveal that the binding of [Ala^{13}]Ang(1–21) to the S-protein is about 50-fold weaker than that of Ang(1–21) while [Ala^{114}]Ang(108–123) binds to RNase(1–118) about 1.5-fold more weakly than the parent peptide (data not shown). These results indicate that complex formation would have been at least 50% under the assay conditions employed. Therefore, His-13 and His-114 must both be required for catalysis by the hybrid enzymes.

Discussion

In recent years significant advances have been made in the isolation and characterization of angiogenic substances [for reviews see Vallee et al. (1985) and Folkman and Klagsbrun (1987)]. Initial efforts employed various tumor cell lines as a source of angiogenic molecules, primarily because of the presumed involvement of such substances in tumor neovascularization. However, it is now evident that many of the angiogenic proteins currently known are present in normal tissue and/or body fluids, thereby implicating these materials in "normal"—as well as "tumor"—angiogenesis. The elucidation of the chemical mechanism(s) by which these agents act and how their activity is controlled in vivo is an area of active research.

Previous reports from this laboratory have described the primary structure and gene sequence of human angiogenin (Strydom et al., 1985; Kurachi et al., 1985). Initially isolated from media conditioned by an HT-29 colon carcinoma cell line (Fett et al., 1985), angiogenin has also been found in normal human plasma (Shapiro et al., 1987) and, hence, is not tumor specific. We have emphasized the striking sequence homology between angiogenin and the pancreatic RNases (Strydom et al., 1985). The overall structures of the two proteins also appear to be quite similar. Angiogenin contains counterparts of virtually every residue thought to be involved in the enzymatic activity of RNase A, i.e., the catalytic residues His-12, Lys-41, and His-119 and the substrate binding residues Gln-11, Thr-45, Glu-111, Asp-121, and Ser-123. Moreover, comparison of the primary structure of angiogenin with the sequences of all known pancreatic RNases shows an overall sequence similarity of 69%. The putative three-dimensional structure of the backbone of angiogenin computed by energy-minimization procedures superimposes readily on that of bovine RNase A in the regions of regular structure (Palmer et al., 1986). The predictive capacity of this homology is indicated by the finding that angiogenin is a ribonucleolytic enzyme (Shapiro et al., 1986a) and that it binds tightly to a ribonuclease inhibitor from human placenta (Shapiro & Vallee, 1987).

Although the physiological substrate(s) of angiogenin is (are) not identified as yet, the fact that it possesses ribonucleolytic activity suggests that its role in angiogenesis may involve RNA. The simultaneous abolition of its enzymatic and angiogenic activities either by chemical modification of histidine residues with bromoacetate (Shapiro et al., 1986a) or by addition of placental ribonuclease inhibitor (Shapiro & Vallee, 1987) suggests that the two activities are interdependent. Elucidation of the structural features of angiogenin critical to its enzymatic function could therefore provide further insight into its mechanism of action in vivo.

The use of angiogenin/RNase hybrid enzymes—wherein particular regions of primary structure in RNase are replaced by the corresponding segments in angiogenin (or vice versa)—provides a means to identify the components of angiogenin that are responsible for its characteristic activities. This approach explores the functional contribution of both overall structure and conserved residues.

Segments of angiogenin containing putative catalytic and/or substrate binding residues constitute prime candidates for constructing such hybrid enzymes. The logical counterparts are inactive proteolytic fragments of RNase A, which are known to associate with natural or synthetic peptides of RNase to generate enzymatically active complexes. RNase S is such

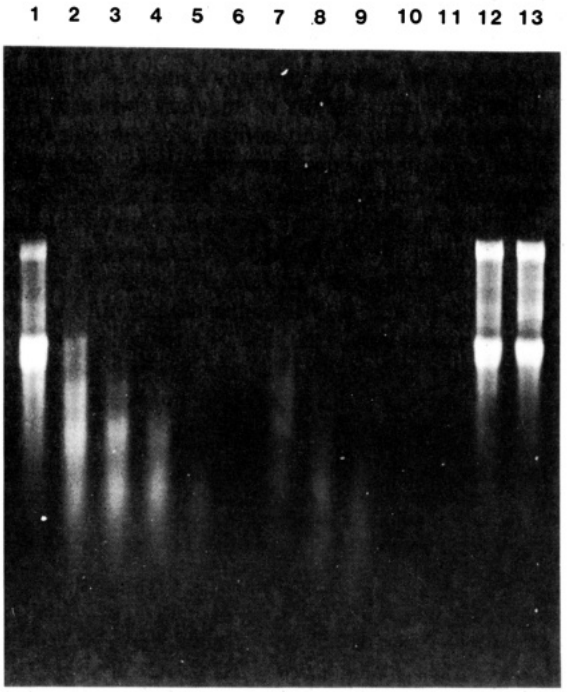

FIGURE 5: Time course for degradation of 18S and 28S rRNA by RNase(1–118)/Ang(108–123) hybrid enzymes. Assays were performed as described in Figure 4. (Lane 1) Control RNA incubated with 1.5 nM RNase(1–118) for 60 min; (lanes 2–6) samples containing 50 μM Ang(108–123) and 1.5 nM RNase (1–118) incubated for 5, 10, 15, 30 and 60 min, respectively; (lanes 7–11) samples containing 50 μM [Phe[115]]Ang(108–123) and 1.5 nM RNase(1–118) incubated for 5, 10, 15, 30, and 60 min, respectively; (lane 12) sample containing 50 μM Ang(108–123) incubated for 60 min; (lane 13) sample containing [Phe[115]]Ang(108–123) incubated for 60 min.

an active complex of S-peptide and S-protein. It is formed by cleavage of RNase A at the Ala-20–Ser-21 bond (Richards, 1958; Richards & Vithayathil, 1959). Complexes derived from RNase(1–118) and synthetic C-terminal peptides such as RNase(111–124) are also enzymatically active (Gutte et al., 1972). The structural features responsible for formation of these active complexes have been investigated in detail by a variety of semisynthetic and X-ray crystallographic means [reviewed in Richards and Wyckoff (1971) and Blackburn and Moore (1982)].

The present study uses these systems to examine the capacity of synthetic N- and C-terminal angiogenin peptides to fulfill the function of the equivalent portions of RNase.[4] Many of the residues involved in binding and catalysis are located in these structural segments of RNase. The more important ones, their putative roles, and the corresponding residues in angiogenin are listed in Table III. The amino acid sequences within these two segments of angiogenin differ considerably from their equivalent regions in RNase A (Figure 1). For RNase, the N- and C-terminal peptides provide the catalytic histidine residues as well as several important interactions with substrate. Comparison of the N-terminal segments of the two proteins shows that only four of the first twenty residues are conserved (Figure 1A); however, these four residues are fully conserved among the pancreatic RNases (Beintema et al., 1986), and residues 15–20 in the S-peptide are not required for binding or activity (Potts et al., 1963). The structures of the C-terminal regions are also quite different (Figure 1B). In particular, there is a two amino acid deletion in angiogenin at the positions corresponding to residues 114 and 115 in RNase, and it has a four-residue extension at its C-terminus. Despite these differences, the N- and C-terminal angiogenin peptides are able to combine with the S-protein and RNase-(1–118), respectively, to give active hybrid ribonucleases. The activities of the Ang(1–21)/S-protein complex range from about 50% with C>p and CpA to 7% with UpG (Table I). The activity found with RNase(1–118)/Ang(108–123) toward cyclic nucleoside monophosphates and dinucleoside phosphates was substantially lower than that found with the N-terminal hybrids (Table II). In all cases, however, the activities of these hybrid enzymes were several orders of magnitude higher than that of angiogenin itself with these substrates (Shapiro et al., 1986a, 1987). When alanine replaces His-13 and His-114 in Ang(1–21) and Ang(108–123), respectively, the hybrid complexes are inactive, consistent with a specific functional role for these residues.

It was suggested previously that Phe-120 in RNase A may play an important role in aligning His-119 into an optimal position for catalysis (Table III) but that it is not involved directly in substrate binding (Lin et al., 1972). In angiogenin, this position is occupied by leucine (Leu-115; Figure 1B). The replacement of Leu-115 in Ang(108–123) by Phe results in a 14–100-fold increase in activity toward the substrates examined (Table II). Thus Leu-115, in large part, accounts for the relatively low activity of the hybrid enzyme.

The apparent peptide/protein dissociation constants were determined for several of the hybrid enzymes examined by using dinucleoside phosphates as substrate. Comparisons of these values with those available for the binding of RNase peptides to RNase protein fragments indicate the degree of structural complementarity between these portions of angiogenin and RNase. Many of the interactions required for complex formation with RNase are listed in Table III. The apparent dissociation constant for the Ang(1–21)/S-protein complex, 1.0 μM, is within an order of magnitude of that measured for the S-peptide, 0.11 μM (Figure 2). The replacements indicated in Table III likely have bearing on the small difference in the dissociation constants. In addition, replacement of Glu-9 by leucine in the S-peptide increases K_d by 3-fold (Dunn & Chaiken, 1975), suggesting that this substitution in Ang(1–21) may account for as much as one-third of the loss in binding as compared with S-peptide. The apparent dissociation constant for the RNase(1–118)/Ang-(108–123) hybrid (25 μM; CpA as substrate) is 125-fold higher than the value of 0.2 μM reported previously for the binding of RNase(111–124) to RNase(1–118) (C>p as substrate) (Gutte et al., 1972). While many of the residues in RNase(111–124) thought to be important for binding are also present in Ang(108–123) (Table III), there are several striking differences in overall peptide structure, including a two-residue deletion and a positively charged C-terminal region (Figure 1B), which could well influence the binding of the angiogenin peptide to RNase(1–118).

The activities of the hybrid enzymes toward rRNA were assessed in order to facilitate comparison to angiogenin. The polynucleotide cleavage pattern observed with the Ang(1–21)/S-protein complex shows a striking resemblance to the pattern characteristic of angiogenin (Figure 4). However, this cleavage pattern is obtained with hybrid enzyme concentrations ~1000-fold less than that required with angiogenin. These findings are in marked contrast to those with the RNase(1–

[4] Preliminary experiments have shown that a variety of proteases—including subtilisin and pepsin—do not cleave angiogenin selectively. Therefore, angiogenin protein fragments analogous to either RNase S-protein or RNase(1–118) are not available, and experiments involving the hybrid enzymes prepared from RNase peptides and angiogenin protein fragments cannot yet be performed.

Table III: Proposed Roles of Some Residues near the N- and C-Termini of RNase A and the Corresponding Amino Acid in Angiogenin

RNase A residue	proposed role(s)	angiogenin residue
	N-Terminus[a]	
Glu-2	side-chain carboxyl H bonds with Arg-10 to stabilize α-helix	Asn-3
Phe-8	hydrophobic interaction with S-protein core	Phe-9
Arg-10	side-chain guanidino group H bonds to Glu-2	Thr-11
Gln-11	side-chain amide H bonds to phosphate of substrate	Gln-12
His-12	carbonyl H bonds to NH of Val-47; removes 2′OH proton from substrate during catalysis	His-13
Met-13	hydrophobic interaction with Val-47, Leu-51, and Val-54	Tyr-14
Asp-14	side-chain carboxyl H bonds to phenol of Tyr-25 and/or guanidino group of Arg-33; NH H bonds to carbonyl of Val-47; terminates α-helix	Asp-15
	C-Terminus[b]	
Val-118	NH H bonds to carbonyl of Ala-109	Val-113
His-119	carbonyl H bonds to NH of Ala-109, protonates 5′O during catalysis	His-114
Phe-120	maintains alignment of His-119	Leu-115
Asp-121	carboxyl H bonds to imidazole of His-119; NH H bonds to carbonyl of Ile-107	Asp-116
Ala-122	carbonyl H bonds to NH of Ile-107	Gln-117
Ser-123	side-chain hydroxyl H bonds to uracil	Ser-118

[a] Proposed roles were compiled from X-ray crystallographic studies (Richards & Wyckoff, 1973; Wlodawer et al., 1982; Brünger et al., 1985) and from chemical studies reviewed elsewhere (Richard & Wyckoff, 1971; Blackburn & Moore, 1982). [b] Proposed roles were compiled from X-ray crystallographic studies (Richards & Wyckoff, 1973; Wlodawer et al., 1982) and from chemical studies summarized in Blackburn and Moore (1982).

118)/Ang(108–123) hybrid where the rRNA cleavage pattern is similar to that produced by RNase A (Figure 5, lanes 2–6).

The data provide insight into the enzymatic activity differences between angiogenin and RNase. Thus, the N-terminal region of angiogenin in combination with the appropriate RNase core fragment is catalytically active toward conventional RNase substrates. This would imply that structural features contained within this region are not responsible for the absence of conventional pancreatic RNase activity with angiogenin. In contrast, studies with the C-terminal hybrids indicate that structural features contained near the C-terminus, which include the Phe → Leu substitution at position 115, do lead to decreased conventional RNase activity. Moreover, the results with rRNA as substrate imply that the Ang(1–21)/S-protein complex retains features of both parent enzymes. It initially generates cleavage products that are similar in kind to those produced by angiogenin, but these are largely degraded with time by increased levels of the hybrid enzyme. This finding points to an important role for the N-terminal region of angiogenin in conferring substrate selectivity.

This approach can be extended to other regions of angiogenin and could provide additional information concerning its structure and function, particularly with regard to angiogenic activity. Preliminary assays indicate that mixtures of Ang(1–21) and S-protein are not angiogenic in the chorioallantoic membrane assay (unpublished data). However, a rigorous assessment of the angiogenic potential of such hybrids most likely requires preparation of covalent derivatives by either chemical or genetic means. The availability of the angiogenin gene (Kurachi et al., 1985) should allow the construction of a wide variety of covalent angiogenin/RNase hybrids through recombinant DNA techniques.

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DNA Binding Domain of *Escherichia coli* DNA Polymerase I: Identification of Arginine-841 as an Essential Residue†

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ABSTRACT: To identify the DNA binding site(s) in *Escherichia coli* DNA polymerase I (pol I) (Klenow fragment), we have used an active-site-directed reagent, phenylglyoxal (PG), which specifically reacts with arginine residues. Preincubation of DNA pol I with PG resulted in the loss of polymerase, 3′–5′-exonuclease, and DNA binding functions. Furthermore, the presence of DNA but not deoxynucleoside triphosphates protected the enzyme from inactivation. Labeling studies with [7-^{14}C]PG indicated that two arginine residues were modified per mole of enzyme. In order to locate the site of PG modification, we digested the PG-treated enzyme with trypsin and V-8 protease. The resulting peptides from each digest were then resolved on reverse-phase hydrophobic columns. An appearance of a new peptide peak was observed in both tryptic and V-8 protease digests. Since inclusion of template-primer during PG modification of enzyme blocks the appearance of these peaks, these peptides were concluded to represent the template-primer binding domain of pol I. Indeed, the extent of inactivation of enzyme by PG treatment correlated very well with the quantitative increase in the new tryptic peptide peak. Amino acid composition analysis of both tryptic peptide and V-8 peptide revealed that the two peptides were derived from the same general region; tryptic peptide spanned between residues 837 and 857 while V-8 peptide spanned between residues 841 and 870 in the primary sequence of pol I. Sequence analysis of tryptic peptide further identified arginine-841 as the site of PG modification, which implicates this residue in the DNA binding function of pol I.

Escherichia coli DNA polymerase I is a monomeric protein (M_r 103K) with three distinct enzyme activities, viz., polymerase, 5′–3′-exonuclease, and 3′–5′-exonuclease. Mild proteolysis cleaves the protein into two separate domains: the large C-terminal fragment [67 kilodaltons (kDa)][1] retains the polymerase and 3′–5′-exonuclease activities while the smaller N-terminal fragment harbors the 5′–3′-exonuclease activity (Brutlag et al., 1969; Klenow & Henningson, 1970). The gene for DNA Pol I has been cloned and the primary structure of the protein determined (Joyce et al., 1982; Brown et al., 1982). Recently, the crystal structure of the large fragment (Klenow) has also been elucidated (Ollis et al., 1985). All these studies together with the early kinetic studies (Englund et al., 1969; Huberman & Kornberg, 1970; Que et al., 1978) have provided some insight into the possible arrangement of the different domains which participate in the expression of the various enzyme activities of pol I.

Studies in our laboratory are aimed at defining the structure–function relationships in DNA polymerases by utilizing various active-site-directed reagents (Srivastava & Modak, 1980a,b, 1982, 1983; Srivastava et al., 1983; Abraham & Modak, 1984). We have been able to define some of the structural domains of DNA polymerases which are responsible for the binding of substrate dNTPs and template-primer. Pyridoxal 5′-phosphate, which has been shown to be a substrate binding site directed reagent (Modak, 1976; Modak & Du-

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[1] Abbreviations: pol I, *Escherichia coli* DNA polymerase I; PG, phenylglyoxal; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; dNTP, deoxynucleoside triphosphate; HPLC, high-performance liquid chromatography; TPCK, tosylphenylalanine chloromethyl ketone; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; PTH, phenylthiohydantoin; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane.

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