Importance of Asparagine-61 and Asparagine-109 to the Angiogenic Activity of Human Angiogenin†

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ABSTRACT: Two distinct regions of angiogenin are critical for angiogenic activity: a catalytic site capable of cleaving RNA and a noncatalytic site, encompassing residues 60–68, which may bind to an endothelial cell-surface receptor [Hallahan, T. W., Shapiro, R., & Vallee, B. L. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88,* 2222–2226]. We have now shown that Asn-61 is an essential residue within the cell-binding site and that in addition a segment containing Asn-109 is part of this site. Both asparagines undergo nonenzymatic deamidation during long-term storage or treatment at alkaline pH. While the isolated desamido-61 and desamido-109 derivatives retain nearly full enzymatic activity, their angiogenic activity on the chicken embryo chorioallantoic membrane is markedly attenuated and they do not inhibit angiogenin-induced neovascularization. Tryptic peptide mapping and Edman degradation demonstrate that the isolated deamidated derivatives primarily contain isoaspartic rather than aspartic acid at the positions in question (83% for desamido-61, >99% for desamido-109). Aspartic acid replacement of Asn-61 and Asn-109 by site-directed mutagenesis results in the same ribonucleolytic and angiogenic activities as those of the spontaneous deamidation products. However, the aspartyl derivatives differ strikingly from their isoaspartyl counterparts in that they do inhibit angiogenin-induced angiogenesis. These results indicate that the combination of ribonucleolytic activity and receptor-binding capacity is not sufficient for angiogenic activity and that Asn-61 and Asn-109 within the noncatalytic site are required for some additional function, as yet undefined. They further imply that the overall structure in the region of these two asparagines, which would be altered by the lengthening of the polypeptide backbone, is critical for receptor binding.

Human angiogenin, a 14.1-kDa monomeric protein, was originally identified by virtue of its capacity to induce neovascularization on the chicken embryo chorioallantoic membrane (CAM)[1] (Fett et al., 1985). It was later found to be a member of the pancreatic ribonuclease superfamily (Strydom et al., 1985; Kurachi et al., 1985). It exhibits a unique ribonucleolytic activity (Shapiro et al., 1986b) that is critical for its angiogenic activity: chemical modification (Shapiro et al., 1986b), inhibitor binding (Shapiro & Vallee, 1987), or site-directed mutagenesis (Shapiro et al., 1989, Shapiro and Vallee, 1989) at the enzymatic active site all abolish both activities simultaneously. In addition, a region of angiogenin distinct from the catalytic site is also necessary for angiogenesis, likely as part of a receptor-binding site. Proteolytic cleavage within this region, encompassing residues 60–68, substantially decreases angiogenic but not enzymatic activity (Hallahan et al., 1991). These findings have led to the proposal of a dual-site model in which both a catalytic site and a cell-binding site are required for the organogenic activity of angiogenin.

We have now identified Asn-61 as an essential residue within the putative receptor-binding site. Its spontaneous deamidation markedly attenuates angiogenesis but does not appreciably affect ribonucleolytic activity. Deamidation of Asn-109 similarly separates the enzymatic and angiogenic activities of the protein, indicating that the portion of primary structure encompassing this residue also forms part of the cell-binding site. According to the calculated three-dimensional structure of angiogenin (Palmer et al., 1986), residue 109 lies in the same region of the molecule as Asn-61, adjacent to the cell-binding segment identified by specific proteolysis. Both of these deamidation reactions are nonenzymatic and are induced by long-term storage or treatment at alkaline pH. In each instance isoaspartic acid is the primary deamidation product, introducing an additional methylene group into the polypeptide backbone. Mutagenic replacement of each of the two asparagine side chains by aspartic acid yields derivatives that have angiogenic and ribonucleolytic properties similar to those of the isoaspartyl products but differ strikingly in terms of their capacity to inhibit angiogenin-induced neovascularization. These results confirm and extend the dual-site model for the action of angiogenin (Hallahan et al., 1991).

EXPERIMENTAL PROCEDURES

Materials. Angiogenin was obtained from human plasma (Shapiro et al., 1987a) or from recombinant expression systems in either mammalian cells (Kurachi et al., 1988) or *Escherichia coli*[2] (Shapiro et al., 1988). Protein concentrations were

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[1] Abbreviations: CAM, chorioallantoic membrane; HPLC, high-performance liquid chromatography; C18, octadecylsilane; TFA, trifluoroacetic acid; PCR, polymerase chain reaction; PTH, phenylthiohydantoin; CpA, cytidylyl-3′,5′-adenosine; CpG, cytidylyl-3′,5′-guanosine; UpA, uridylyl-3′,5′-adenosine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Mes, 2-morpholinoethanesulfonic acid; IL, interleukin; bFGF, basic fibroblast growth factor.

[2] Angiogenin produced in *E. coli* differs from that isolated from human plasma and mammalian cells only with respect to its N terminus: Met(−1) vs pyroglutamic acid-1, respectively. This difference does not affect enzymatic or angiogenic activity (Shapiro et al., 1988).

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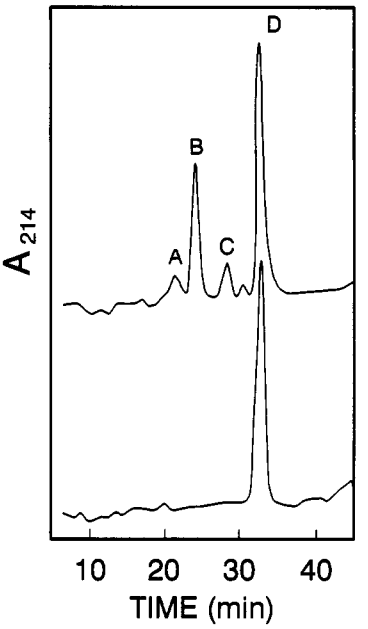

FIGURE 1: Mono S cation-exchange chromatography of a freshly isolated angiogenin sample (lower trace) and a sample stored for 2 years at pH 8 (upper trace). Elution was performed with a 50-min linear gradient of 0.15–0.55 M NaCl in 10 mM Tris, pH 8.0, at a flow rate of 0.8 mL/min at ambient temperature.

determined by amino acid analysis. The expression plasmids pAng1 and pAng2 were from a previous study (Shapiro et al., 1988). Endoproteinase Glu-C (V8) was purchased from Boehringer Mannheim. Sources of other materials have been listed previously (Shapiro et al., 1986a,b, 1987a).

Isolation of Deamidated Angiogenin Derivatives. Deamidated derivatives of angiogenin were separated from native angiogenin by Mono S HPLC as described in the legend to Figure 1 and were further purified on a Synchropak C18 HPLC column (Synchrom Inc., 250 × 4.5 mm). Elution was achieved with a 90-min linear gradient of 30–50% solvent B at 0.8 mL/min at ambient temperature, where solvent A was 0.1% TFA in water and solvent B was 2-propanol/acetonitrile/water (3:2:2) containing 0.08% TFA. Peak fractions were pooled, diluted 1:1 with water, lyophilized, and reconstituted in water prior to use for structural studies and measurements of angiogenic and enzymatic activity.

Oligonucleotide-Directed Mutagenesis. Genes encoding angiogenin with an aspartic acid residue replacing either Asn-61 or Asn-109 were obtained by a modification of the overlap extension method of Ho et al. (1989). Oligonucleotides were synthesized on a Biotix Model 102 DNA synthesizer. The complementary mutagenic oligonucleotide primers, designated b and c, for N61D were pCGGGTTACC*GTC*TTTGTTTTC and pGAAAACAAA*GAC*GGTAACCCG and for N109D were pTGGCAGACC*GTC*TTCACAAGC and pGCTTGTGAA*GAC*GGTCTGCCA. The 5′ flanking primer, designated a, used in both cases was pAACTAGTACGCAAGTTCA and the 3′ flanking primer, designated d, was pTCGACGGATCCCCGGGAATTC. The template for PCR extension of the primers was the plasmid pAng2 that contained a synthetic angiogenin gene (Shapiro et al., 1988). PCR reactions were performed with a GeneAmp DNA amplification kit (Perkin-Elmer Cetus) according to the manufacturer's specifications. Fragments generated by the first set of PCR reactions with primers a plus b and c plus d were purified after agarose gel electrophoresis by means of a US Bioclean kit (United States Biochemical). The two products, containing overlapping segments, were then mixed and subjected to PCR amplification with the two flanking primers a and d. The fusion product generated was digested with *Kpn*I and *Eco*RI, purified by electrophoresis on a 2% low-melting-point agarose gel (NuSieve GTC, FMC Bioproducts) and inserted into *Kpn*I/*Eco*RI-digested pAng1 by standard procedures. *E. coli* W3110 cells were then transformed with the resultant plasmid. The coding region of this plasmid was sequenced in its entirety to ensure the presence of the desired mutation and the absence of spurious mutations.

Expression and Isolation of N61D- and N109D-Angiogenin. Mutant proteins were expressed and purified to homogeneity as described (Shapiro et al., 1988). Material eluting from the C18 HPLC column was diluted 1:1 with water, lyophilized, and reconstituted in water.

Structural Characterization of Angiogenin Derivatives. Amino acid analysis, amino terminal sequencing, and digestion with trypsin were performed as described (Strydom et al., 1985). Digestion of N61D-angiogenin (12 μM) with endoproteinase Glu-C (V8) (5% w/w) was performed for 18 h at 37 °C in 50 mM ammonium bicarbonate. Proteolytic digests were applied to a Synchropak C18 column, and elution of peptides was achieved with a gradient of 10–50% solvent B (tryptic digests) or 2–50% solvent B (V8 digest) in 90 min at a flow rate of 1 mL/min at ambient temperature, where solvents were as described above. One-minute fractions were collected. For the tryptic maps, material not retained on the initial C18 column, containing peptides T1–T5 and T13, was partially dried under nitrogen and rechromatographed on a Beckman Ultrasphere C18 column with a gradient of 1–15% solvent B in 60 min at a flow rate of 1 mL/min, where solvent A was 0.1% TFA in water and solvent B was 70% acetonitrile containing 0.08% TFA. One-minute fractions were collected.

Assays. Activities toward yeast tRNA (Shapiro et al., 1987b), dinucleoside 3′,5′-phosphates (Shapiro et al., 1986a,b; Harper & Vallee, 1989), and 18S and 28S rRNA (Shapiro et al., 1986b) were measured as described. Angiogenic activity was assessed on the chicken embryo CAM (Knighton et al., 1977; Fett et al., 1985). The number of positive and negative responses for each sample on 32–67 eggs was determined and χ^2's were calculated from an outcome contingency table comparing the test sample with a water control; the associated probabilities, p, were then obtained. A value of $p < 0.05$ is needed for a sample to be considered active.

RESULTS

Isolation of Deamidated Angiogenin Derivatives. Human angiogenin can be purified to homogeneity from a variety of natural and recombinant sources by CM-52 cation-exchange chromatography followed by Mono S HPLC (Shapiro et al., 1987a; Kurachi et al., 1988). Rechromatography of the freshly isolated final product on a Mono S column yields a single protein peak (Figure 1, lower trace). However, rechromatography of this material after it had been stored at 4 °C for prolonged times in the column elution buffer (10 mM Tris, 0.35 M NaCl, pH 8) reveals four peaks (Figure 1, upper trace): one (peak D) has the normal retention time of angiogenin and three (peaks A–C) elute 12.3, 9.3, and 4.8 min earlier. In the example shown, storage was for 2 years and components A–C comprise about 35% of the total. Angiogenin samples stored in water for comparable periods contain several-fold lower amounts of the faster eluting components. Samples stored at pH 2 (40% C18 HPLC solvent B; see Experimental Procedures) undergo no detectable conversion.

Derivatives eluting at the same times as peaks A–C are also generated during relatively short incubations of angiogenin

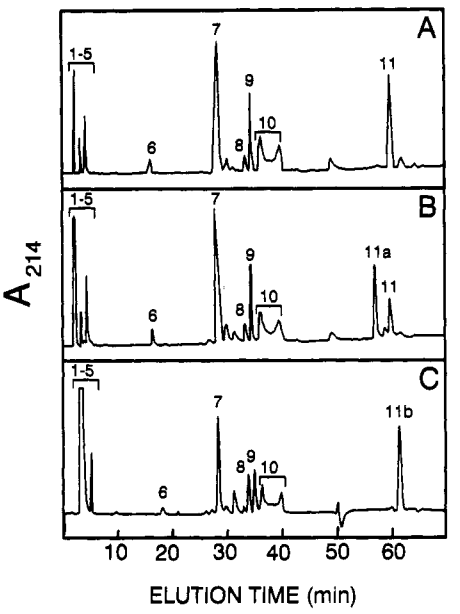

FIGURE 2: Fractionation of tryptic digests of angiogenin (A), Mono S peak B angiogenin derivative (B), and Mono S peak C angiogenin derivative (C) on a Synchropak C18 HPLC column. Chromatography conditions are detailed under Experimental Procedures. Peptide designations are from Strydom et al. (1985) except for the new peptides 11a and 11b.

at 22 or 37 °C under alkaline conditions. The combined rate of formation of products A–C at 37 °C is 4%/day at pH 8 (50 mM Tris) and ~80%/day at pH 10 (100 mM NH_4HCO_3).

Components B–D were purified further by C18 HPLC for structural and functional characterization. Derivative B elutes 1.25 min later than angiogenin, whereas derivative C elutes 3.24 min earlier. Component D elutes at the same time as angiogenin. Peak A appeared to be heterogeneous and was generally obtained in lower yield than B and C; it was not examined further.

Structural Characterization of Deamidated Derivatives. Components B–D are indistinguishable from unmodified angiogenin both by SDS–PAGE under reducing conditions and by amino acid analysis. The chromatographic properties of component D are identical to those of angiogenin, indicating that it is unmodified protein. This was confirmed by tryptic peptide mapping, which revealed the same pattern as for angiogenin.

Both the decreased cation-exchange HPLC retention times of components B and C as compared with angiogenin and the increase in their rate of production with pH suggested that they might represent deamidated derivatives. Peptide mapping and sequence analysis results support this conclusion. The tryptic peptide map of product B (Figure 2B) is identical to that of angiogenin (Figure 2A) except that the yield of peptide T11 (amino acids 55–60 linked by disulfide bond to amino acids 102–121) is low (~35%) and a new peptide, T11a, is obtained that elutes 2.7 min before T11 [see Strydom et al. (1985) for a description of angiogenin tryptic peptides]. The amino acid composition of peptide T11a (not shown) reveals the presence of angiogenin sequences normally contained in peptide T11 (amino acids 55–60 and 102–121) plus that contained in peptide T4a (61–66), indicating that trypsin did not cleave at Lys-60. Amino terminal sequencing of peptide T11a (Figure 3) yielded two sequences, one beginning at Ala-55 and the other at Asn-102. The yield of the latter sequence was relatively high through 10 cycles. For the former sequence, however, no PTH-amino acid was obtained at cycle 7 and

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Desamido-61     Ala-Ile-Cys-Glu-Asn-Lys-Asn-Gly-
Peptide T11a    214 227  62  72  55  73  <1  <1
                     └──────────────────┘
                Asn-Val-Val-Val-Ala-Cys-Glu-Asn-Gly-Leu
                106 230 219 207 174  67  90  49  67 103

Desamido-109    Ala-Ile-Cys-Glu-Asn-Lys
Peptide T11b    292 221  +  114 104  29
                     └──────────────────┘
                Asn-Val-Val-Val-Ala-Cys-Glu-Asn-
                188 279 279 258 283  +  103  <1
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FIGURE 3: Sequencer results for tryptic peptides of deamidated angiogenin derivatives. Desamido-61 angiogenin peptide 11a, representing disulfide-linked amino acids 55–66 and 102–121, was from the digest shown in Figure 2B. Desamido-109 angiogenin peptide 11b, representing disulfide-linked amino acids 55–60 and 102–121, was from the digest shown in Figure 2C. The established sequence is given (Strydom et al., 1985), with the yield of amino acid residues at each cycle, in picomoles, given below the residue. Cycle 7 for the desamido-61 angiogenin peptide and cycle 8 for the desamido-109 derivative both yielded <1 pmol of PTH-Asp.

thereafter, suggesting that Asn-61 was deamidated to isoaspartic acid, since this residue is not released during Edman degradation (Smyth et al., 1962; Groskopf et al., 1966). An isoaspartate at this position would also account for the lack of cleavage by trypsin, which does not readily hydrolyze peptide bonds adjacent to such residues (Di Donato et al., 1986). As noted below, trypsin effectively cleaves at Lys-60 when residue 61 is a normal aspartic acid, as in the N61D mutant. We therefore conclude that the ~65% of component B not cleaved by trypsin at Lys-60 contains isoaspartic acid at position 61.

In order to identify the amino acid at position 61 in the ~35% of component B which was cleaved by trypsin, the additional peptide(s) containing this residue had to be isolated. In unmodified angiogenin, amino acid 61 is the N-terminal residue of tryptic peptide 4a, which elutes together with several other small peptides (T1–T5) in the unretained fractions during standard mapping procedures (Figure 2A). These fractions for component B (Figure 2B) were therefore rechromatographed on a C18 HPLC column (see Experimental Procedures), and the eluted peptides were subjected to amino acid analysis. The amino acid compositions of two peptides, separated by 1–2 min, were identical to that of T4a. Edman degradation yielded PTH-aspartic acid for the later but no PTH-amino acid for the earlier peptide,[3] indicating the presence of an N-terminal isoaspartic acid. The relative distribution of the isoaspartic acid and aspartic acid peptides was ~1.2:1. Thus, overall, angiogenin derivative B is a mixture of deamidation products containing isoaspartic acid and aspartic acid at position 61 in a ~5:1 ratio.

The tryptic peptide map of component C (Figure 2C) is identical to that for angiogenin except that peptide T11 is absent and a new peptide, T11b, is obtained which elutes 1.8 min later and has an amino acid composition identical to that of native peptide T11. Amino terminal sequencing revealed two sequences, corresponding to residues 55–60 and 102–108 (Figure 3). The yield dropped precipitously, from 103 to <1 pmol, after cycle 7, consistent with the presence of an isoaspartate residue at position 109.[3] Any peptide containing a normal aspartic acid at this position would have been present in the same column fractions that were sequenced (peptide T11b of N109D-angiogenin, described below, elutes at the same time as the isoaspartyl peptide). We conclude that component C represents angiogenin deamidated at Asn-109, with the final C18-purified derivative exclusively containing isoaspartate rather than aspartate at this position.

[3] Edman degradation of native angiogenin yields a substantial amount of PTH-asparagine at this cycle.

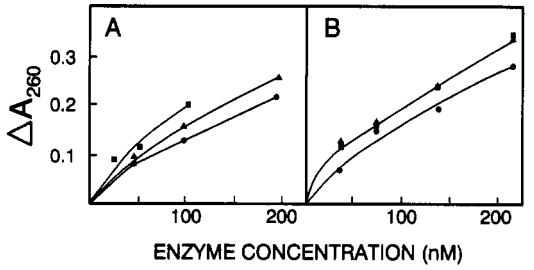

FIGURE 4: Cleavage of tRNA: (A) angiogenin (■), desamido-61 angiogenin (●), and desamido-109 angiogenin (▲); (B) angiogenin (■), N61D-angiogenin (●), and N109D-angiogenin (▲). Assays were performed as described (Shapiro et al., 1987b) except that incubations were for 2 h at 37 °C.

Table I: Activity of Deamidated Angiogenin Derivatives toward Dinucleoside 3′,5′-Phosphates[a]

	k_{cat}/K_m, M^{-1} s^{-1}		
		derivative[b]	
NpN′	angiogenin[c]	desamido-61	desamido-109
CpA	12.0	10.4	13.3
CpG	4.1	2.4	3.8
UpA	0.6	5.1	1.2

[a] Incubations were performed at 37 °C in 33 mM Mes (pH 6.0), 33 mM NaCl, containing 0.1 mM substrate, and 7.0 μM enzyme. At various times, aliquots (15–20 μL) were applied to a Waters Radial-PAK C18 column for quantitation of substrate and products (Shapiro et al., 1986a,b). Values for k_{cat}/K_m = ln ($[S]_0/[S]_t$)/t[E] where $[S]_0$ and $[S]_t$ are the substrate concentrations at the initial time and time t, respectively, and [E] is the enzyme concentration. [b] Derivatives were formed from angiogenin expressed in *E. coli* (Shapiro et al., 1988). Desamido-61 angiogenin is a ~5:1 mixture of isoAsp-61 and Asp-61 angiogenins. Desamido-109 angiogenin contains >99% isoaspartate at position 109. [c] Values for angiogenin are from Hallahan et al. (1991).

Enzymatic Activity of Deamidated Derivatives. Desamido-61 and desamido-109 angiogenin retain essentially full activity toward tRNA (Figure 4A), the dinucleotides CpA and CpG (Table I), and 18S and 28S rRNA (not shown). The desamido-61 derivative is several-fold more active toward UpA than is either the native or desamido-109 protein (Table I). Both derivatives cleave 18S and 28S rRNA to the same characteristic 100–500 nucleotide fragments as angiogenin (Shapiro et al., 1986b), indicating unchanged selectivity toward this polynucleotide substrate.

Angiogenic Activity of Deamidated Derivatives. Two preparations of the desamido-61 and desamido-109 derivatives were each tested for angiogenic activity on the chick embryo CAM (Table II). One pair was derived from plasma angiogenin which had been stored at pH 8, 4 °C, for 3.5 years; the other was from angiogenin produced in *E. coli* and deamidated by incubation at room temperature for 2 weeks. In both cases the activity of the two derivatives is reduced drastically compared to native angiogenin. Indeed, the percent positive response to 10 ng of each desamido derivative does not differ significantly from that obtained with water controls. In contrast, the material that elutes at the normal retention time of angiogenin (peak D, Figure 1) after storage at 4 °C for 3.5 years has activity comparable to that of freshly prepared angiogenin.

Angiogenesis Inhibition by Deamidated Derivatives. A 5-fold molar excess of either deamidated derivative does not inhibit angiogenin-induced angiogenesis on the chick CAM (Table III). Thus, the percent positive response to angiogenin in the presence of desamido-61 angiogenin (45%) or desamido-109 angiogenin (47%) does not differ significantly from that obtained with angiogenin alone (53%). The amount of unmodified angiogenin employed in these assays is in that region of the dose–response curve where an activity decrease would be most readily apparent (Fett et al., 1985; Kurachi et al., 1988).

Table II: Effect of Deamidation on Angiogenic Activity of Angiogenin[a]

sample	% positive (n)[b]	p[c]
experiment 1[d]		
angiogenin	50 (36)	0.004
desamido-61 angiogenin	18 (51)	>0.5
desamido-109 angiogenin	16 (49)	>0.5
experiment 2[e]		
angiogenin	49 (67)	<0.0001
desamido-61 angiogenin	30 (37)	0.131
desamido-109 angiogenin	20 (40)	>0.5

[a] The CAM assay was employed (Knighton et al., 1977; Fett et al., 1985). A 10-ng aliquot of each test sample was implanted. Data from multiple experiments were combined (12–20 eggs per group). [b] The total number of assays performed for each sample, n, is given in parentheses. [c] Significance, p, was calculated from χ^2's, based on comparison with water control samples tested simultaneously, which produced 17% positive responses (total 65 eggs). A p value of <0.05 must be attained for a sample to be considered positive. [d] Samples denoted angiogenin, desamido-61 angiogenin, and desamido-109 angiogenin represent materials purified by C18 HPLC from peaks D, B, and C, respectively (see Figure 1). In this experiment the angiogenin sample had been stored at 4 °C, pH 8, for 3.5 years. [e] Angiogenin samples employed in this experiment were derived from recombinant protein expressed in *E. coli*. Deamidation was achieved by incubation at pH 8–9, 22 °C, for 2 weeks.

Table III: Angiogenesis Inhibition by Deamidated Angiogenin Derivatives[a]

sample	dose, ng	% positive (n)[b]	p[c]
angiogenin[d]	1	53 (51)	0.0015
desamido-61 angiogenin	5	28 (32)	>0.5
angiogenin + desamido-61 angiogenin	1 + 5	45 (38)	0.028
desamido-109 angiogenin	5	26 (34)	>0.5
angiogenin + desamido-109 angiogenin	1 + 5	47 (34)	0.019

[a] The CAM assay was employed. Data from multiple experiments (12–20 eggs per group) were combined. [b] The total number of assays performed for each sample, n, is given in parentheses. [c] Significance values, p, are based on comparison with water control samples tested simultaneously, which produced a 23% positive response (total 56 eggs). A p value of <0.05 is needed for a sample to be considered active. [d] Angiogenin samples are those employed in experiment 1, Table II.

Preparation of Angiogenin Mutants. Isoaspartyl residues may disrupt three-dimensional structure. Therefore, in order to replace Asn-61 or Asn-109 with an aspartic acid residue, genes encoding N61D- and N109D-angiogenin were produced by oligonucleotide-directed mutagenesis as described under Experimental Procedures. Mutant proteins were expressed in *E. coli* and purified to homogeneity (Shapiro et al., 1988). The N61D- and N109D-angiogenins elute 9.2 and 2.8 min earlier than angiogenin, respectively, on Mono S HPLC whereas both have retention times virtually identical to that of angiogenin on C18 HPLC. The yields were 0.5 and 1.8 mg/L for N61D- and N109D-angiogenin, respectively. Final preparations were >98% pure as judged by SDS–PAGE.

Structural Characterization of Angiogenin Mutants. The amino acid compositions of the two mutant angiogenins are indistinguishable from that of angiogenin, as expected for Asn → Asp substitutions. Tryptic peptide maps indicate that in both cases the three disulfide bonds (in peptides T9, T10, and T11 or T11b) formed correctly and that there are no unexpected changes in structure. Peptide T11b of N109D-angiogenin, which contains residue 109, elutes 2 min later

Table IV: Activity of Angiogenin Mutants toward Dinucleoside 3′,5′-phosphates[a]

	k_{cat}/K_m, M^{-1} s^{-1}		
		mutants	
NpN′	angiogenin[b]	N61D	N109D
CpA	12.0	10.9	13.9
CpG	4.1	3.6	4.4
UpA	0.6	0.6	0.7

[a] Incubations and calculations were performed as described in the legend to Table I. [b] Values for angiogenin are from Hallahan et al. (1991).

Table V: Angiogenesis Inhibition by Angiogenin Mutants[a]

sample	dose, ng	% positive (n)[b]	p[b]
angiogenin	1	47 (57)	<0.001
N61D-angiogenin	5	22 (55)	>0.5
angiogenin + N61D-angiogenin	1 + 5	30 (57)	0.13
N109D-angiogenin	5	25 (53)	0.36
angiogenin + N109D-angiogenin	1 + 5	24 (58)	0.38

[a] See legend to Table III. [b] Significance values, p, are based on comparison with water control samples tested simultaneously, which produced a 17% positive response (total 52 eggs).

than peptide T11 from angiogenin, but the amino acid compositions of the two peptides are the same. The Asn → Asp replacement at position 109 is confirmed by sequencing.

A peptide containing residue 61 was obtained from an endoproteinase Glu-C (V8) digest of N61D-angiogenin. This protease cleaves angiogenin to produce peptide fragments V1 (residues 59–67), V2 (residues 117–123), V3 (residues 109–116), and V4 (residues 68–108 disulfide bonded to residues 1–58) (D. J. Strydom, unpublished results). Peptide V1 isolated from the N61D-angiogenin digest by C18 HPLC has the amino acid composition expected for residues 59–67, and sequencing reveals an aspartic acid residue at position 61.

Enzymatic Activity of Angiogenin Mutants. The enzymatic activities of N61D- and N109D-angiogenin are essentially the same as that of native angiogenin toward tRNA (Figure 4B), dinucleoside phosphates (Table IV), and 18 and 28S rRNA (not shown). The Asp-61 derivative does not exhibit the several-fold increase in k_{cat}/K_m toward UpA that was observed for angiogenin spontaneously deamidated at Asn-61 (Table I), which predominantly contains isoaspartic acid at this position.

Angiogenic Activity of Angiogenin Mutants. The activities of N61D- and N109D-angiogenin in the CAM assay are greatly diminished. Thus, at 5 ng/egg the percent positive response with either mutant is considerably lower than that observed with angiogenin at 1 ng/egg and is indistinguishable from that obtained with water controls (Table V). At 20 ng/egg, the N61D derivative remains inactive ($p = 0.33$; 26% positive response on 39 eggs), whereas the N109D protein is marginally active ($p = 0.05$; 35% positive response on 37 eggs). In contrast to the corresponding spontaneously deamidated derivatives, which primarily contain isoaspartyl residues, both N61D- and N109D-angiogenin inhibit angiogenin-induced angiogenesis: in the presence of 5 ng of the N61D or N109D derivatives, the percent positive response to 1 ng of angiogenin does not differ significantly from that observed with 5 ng of each mutant protein alone (Table V).

DISCUSSION

Asparagine side chains in proteins frequently undergo nonenzymatic deamidation both in vitro and in vivo [see Wright (1991) for a recent review]. Deamidation can occur under mild conditions—neutral or slightly alkaline pH, room temperature—and is a common problem encountered during protein purification and storage. The effects of this conversion on protein function vary. In some instances there appear to be no deleterious consequences, as with human growth hormone (Becker et al., 1988) and mouse epidermal growth factor (DiAugustine et al., 1987). In other cases, however, deamidations lower activity markedly, as with interleukin-1β (Daumy et al., 1991), calmodulin (Johnson et al., 1987), adrenocorticotropin (Graf et al., 1973), lysozyme (Ahern & Klibanov, 1985), and triose phosphate isomerase (Ahern et al., 1987).

The deamidation reaction proceeds primarily via a nucleophilic attack on the asparagine side-chain carbonyl carbon by the peptide bond nitrogen of the C-terminal neighbor, thus forming a cyclic imide intermediate (Bornstein & Balian, 1977; Clarke, 1987). Hydrolysis of this succinimide can then occur via attack on either the α- or the β-carbonyl group to yield either a normal aspartic acid residue or an isoaspartic acid, in which the β-carboxylate is incorporated into the polypeptide backbone and the α-carboxylate becomes a one-carbon side chain. The isoaspartic and aspartic acid products are generally formed in a ratio of ~3:1 (Meinwald et al., 1986; Geiger & Clarke, 1987).

Both primary sequence (Bornstein & Balian, 1977; Stephenson & Clarke, 1989; Wright, 1991) and tertiary structure (Clarke, 1987; Kossiakoff, 1988) are major determinants of the relative rates at which different asparagine residues in proteins undergo deamidation. Asn–Gly sequences are particularly common sites for deamidation, probably due to the lack of steric hindrance during succinimide formation (Bornstein & Balian, 1977). Angiogenin contains two such sequences, at Asn-61 and Asn-109, and these are the ones that undergo deamidation when the protein is maintained at neutral or alkaline pH. The desamido-61 and -109 derivatives can be completely resolved from each other and from the unmodified protein by cation-exchange HPLC (Figure 1). A relatively small quantity of additional, chromatographically distinct, material is also formed during storage (peak A, Figure 1). Although this material was not subjected to structural analysis, its elution time, yield, and peak shape suggest that it may represent angiogenin that has been deamidated at both Asn-61 and Asn-109, which would be a mixture of up to four species having either isoaspartate or aspartate at each position. Alternatively, it may represent products of deamidation at one or more of the remaining seven asparagines, which are followed by leucine, lysine, proline, serine, threonine, or valine (Strydom et al., 1985).

The distribution of isoaspartic and normal aspartic acid in each of the final desamido products isolated, established by tryptic peptide mapping and Edman degradation, differs from the 3:1 ratio that is characteristic of the deamidation reaction. For the desamido-61 derivative, the higher ratio (~5:1) probably reflects partial removal of the Asp-61 form during C18 HPLC, since the Asp-61 (i.e., N61D mutant) protein elutes ~1 min earlier than the bulk of the spontaneously deamidated material. The desamido-109 derivative isolated contained no detectable (<1%) Asp-109 product. In this case, the isoaspartyl and aspartyl (i.e., N109D mutant) proteins separate by 2.0 and 3.2 min during Mono S and C18 HPLC, respectively. Thus, any of the Asp-109 form present should have been removed during purification. Indeed, a small, fairly well-resolved peak with the retention time of the Asp-109 derivative is evident during Mono S HPLC of aged angiogenin (between peaks C and D, Figure 1).

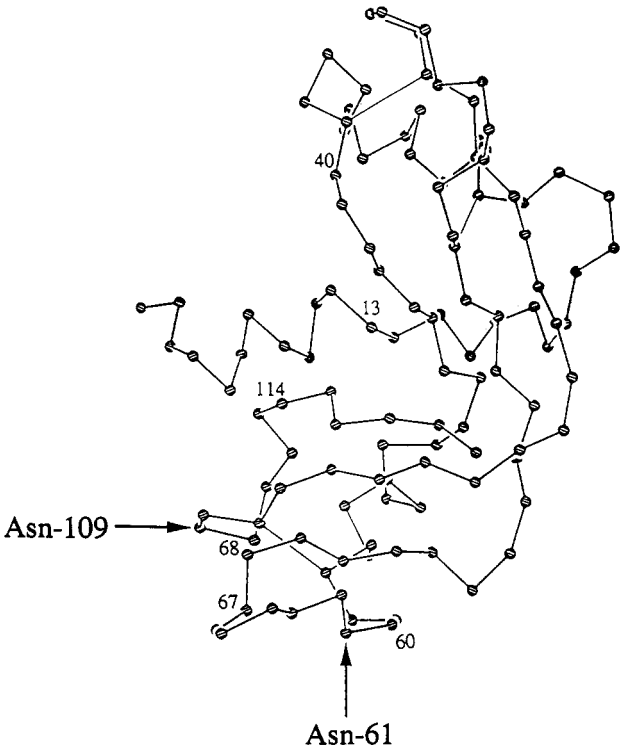

FIGURE 5: Virtual bond (C^{α}···C^{α}) structure of angiogenin calculated by energy minimization procedures (Palmer et al., 1986). Arrows indicate the positions of Asn-61 and Asn-109. The catalytic residues His-13, Lys-40, and His-114 are also indicated.

Both the desamido-61 and desamido-109 angiogenin isolates are enzymatically active; however, neither is capable of inducing neovascularization on the CAM at doses at least 10–20-fold higher than that required for the unmodified protein [see Table II, Fett et al. (1985) and Harper and Vallee (1988)], and neither is an effective inhibitor of angiogenin-induced angiogenesis. The functional consequences of these deamidations are similar to those accompanying earlier modifications of the region of angiogenin containing Asn-61. Thus, proteolytic cleavage of angiogenin at either Lys-60 or Glu-67 (see Figure 5), in each case yielding two disulfide-linked polypeptides, has little effect on enzymatic but abolishes angiogenic activity (Hallahan et al., 1991). Mutagenic replacement of angiogenin residues 58–70 by the corresponding amino acids of the nonangiogenic homologue RNase A (Harper & Vallee, 1989) also greatly attenuates angiogenic, while in this instance actually *increasing* enzymatic activity. Like the desamido-angiogenins, the previous derivatives do not compete efficiently with angiogenin on the CAM (Hallahan et al., 1991). Moreover, the cleaved angiogenins bind much less tightly than the native protein to an endothelial cell-surface protein which may be a component of a putative receptor (Hu et al., 1991).

The properties of the proteolytic derivatives and the angiogenin/RNase hybrid protein, together with the capacity of the catalytically and angiogenically inactive H13A and H114A mutants to effectively inhibit angiogenin-induced neovascularization (Shapiro & Vallee, 1989), formed the basis for a dual-site model for the biological action of angiogenin (Hallahan et al., 1991). According to this model, two sites of angiogenin are essential for angiogenic activity—a catalytic site containing, minimally, His-13, Lys-40, and His-114 and a noncatalytic, cell-binding site encompassing, minimally, residues 60–68. Catalytic activity can be abolished without affecting binding and vice versa but loss of either one abolishes angiogenicity. The present findings also demonstrate the separability of the enzymatic and angiogenic activities of angiogenin and, hence, further support and extend this view. Furthermore, they suggest that within the previously defined cell-binding site a specific residue, Asn-61, is critical and that another segment of primary structure, containing Asn-109, is also involved in cell binding. In this regard, Asn-109 is located in a loop immediately adjacent to that containing residues 60–68 according to the preliminary three-dimensional structure of angiogenin calculated from the X-ray coordinates of RNase A by energy minimization techniques [Figure 5; Palmer et al. (1986)].

Caution must be exercised, however, when drawing conclusions concerning specific roles for Asn-61 and Asn-109 from these data. As discussed above, the desamido derivatives that have been isolated and characterized contain primarily or exclusively isoaspartic rather than aspartic acid at the positions in question. It is possible that in one or both instances the additional methylene group introduced into the polypeptide backbone could disrupt structure such that the functionally detrimental effects of deamidation might not solely reflect the loss of the β-amide group. Indeed, the more than 3 min required to separate the isoAsp-109 derivative from angiogenin during C18 HPLC suggests such a perturbation in this instance.

In order to examine more directly the roles of the two asparagine side chains, each residue was replaced with a normal aspartic acid by site-directed mutagenesis. Like the desamido products, N61D- and N109D-angiogenin have substantially decreased biological activity but unimpaired ribonucleolytic activity. However, both aspartyl derivatives differ strikingly from their isoaspartyl counterparts in that they inhibit angiogenin-induced neovascularization on the CAM and therefore presumably retain a capacity to bind to the receptor. Such binding must be adequate to exclude native angiogenin but not satisfactory for inducing angiogenesis. Thus, the combination of ribonucleolytic activity and cell-binding capacity, although always necessary, is not always sufficient to confer angiogenic activity.

The properties of the various Asp and isoAsp derivatives, in their entirety, fit well into the framework of the proposed dual-site model (Hallahan et al., 1991), in which organogenic activity requires (i) a catalytic site having the potential to cleave RNA or some related substrate and (ii) a noncatalytic site with the capacity to bind to a cell-surface receptor. The present results with these derivatives add important detail to this model and at the same time imply an additional level of mechanistic complexity. Thus, site ii can now be defined more specifically to include Asn-61 as a critical residue within the previously delineated segment 60–68 and to incorporate an adjacent loop containing Asn-109 as well. Moreover, this site now appears to be critical not only for cell binding per se but for additional events, as yet uncharacterized, that are also independent of ribonucleolytic activity, since the Asp-61 and -109 mutants are not angiogenic despite their capacities to cleave RNA and to compete with angiogenin on the CAM. Thus Asn-61 and Asn-109 themselves do not seem to contribute substantially to binding strength. General disruptions of either of the loops containing these residues, produced by proteolysis or conversion to isoaspartate, do result in loss of cell-binding capacity, suggesting that side chains other than those of Asn-61 and Asn-109 and/or the main chain in these segments participate in receptor binding. Consistent with the proposed importance of Asn-61 and Asn-109, both residues are conserved in all five mammalian angiogenins sequenced to date [Bond and Strydom (1989), Bond and Vallee (1990) and D. J. Strydom and M. D. Bond, unpublished results).

Uncouplings of receptor binding and biological activity have been reported previously for other protein effectors. Des-Cys-125 interleukin-2 (IL-2) binds to the high-affinity IL-2 receptor but does not elicit a biological response (Liang et al., 1988). Mutation of Arg-127 in IL-1β to glycine decreases bioactivity markedly while only slightly diminishing receptor binding (Gehrke et al., 1990). Mutation of Lys-145 in the IL-1 receptor antagonist to aspartic acid confers partial agonist activity (Ju et al., 1991). Since the effector proteins in these cases are not thought to have intrinsic enzymatic activity, the functional dissociations observed parallel those obtained with the N61D- and N109D-angiogenin mutants. In none of the prior examples has the molecular basis for uncoupling been defined, although Liang et al. (1988) provided evidence that des-Cys-125 IL-2 may have impaired binding to some additional component of the high-affinity receptor.

Simultaneous binding to two classes of receptor molecules has recently been proposed to be necessary for basic fibroblast growth factor (bFGF) activity (Klagsbrun & Baird, 1991). In this case, binding to heparin or to low-affinity receptors that are cell-surface heparan sulfate proteoglycans (Moscatelli, 1987) is required (Yayon et al., 1991) for binding to high-affinity, specific receptors having intrinsic protein kinase activity (Coughlin et al., 1988). The binding sites for heparin and for the high-affinity receptors appear to reside in at least partially distinct domains of bFGF (Kurokawa et al., 1989). Angiogenin, like bFGF, interacts with two classes of molecules on the cell surface (Badet et al., 1989), and an analogous mechanism may pertain to this protein and account for the observed functional properties of the N61D and N109D mutants. Alternative explanations involving, for example, receptor activation, ligand uptake, or oligomerization, however, cannot be excluded at this time. The use of the two Asp/isoAsp pairs described here and additional mutants in this region, yet to be produced, should be particularly valuable in evaluating these possibilities.

The dual-site model for angiogenin elaborated above is consistent with all present knowledge concerning the protein and its mode of action. It is nonetheless subject to a number of uncertainties and limitations. Evidence for the existence of an angiogenin receptor is, thus far, incomplete. The capacity of various inactive mutant derivatives to inhibit angiogenin in vivo and the isolation of an endothelial cell surface angiogenin-binding protein, both cited above, strongly favor a receptor-based mechanism. So do the activation of second-messenger pathways in vascular endothelial and smooth muscle cells in vitro by angiogenin (Bicknell & Vallee, 1988, 1989; Moore & Riordan, 1990) and the tight, specific binding of angiogenin to endothelial cells (Badet et al., 1989). However, definitive proof, e.g., the demonstration of a role for the angiogenin-binding protein in signal transduction, has yet to be obtained.

In addition, no substrate(s) for angiogenin in vivo has (have) been identified. The proteolytic and deamidated derivatives, although almost fully active toward tRNA and certain dinucleotides, might be less active toward some more physiologically relevant substrate. While the inability of the isoaspartyl and proteolytic derivatives to compete with angiogenin on the CAM would still implicate the region of the protein containing these modifications in receptor binding, the catalytic and cell-binding sites may not be altogether distinct and there may be a more direct interplay between receptor binding and substrate cleavage. Furthermore, it remains possible that loss of enzymatic activity toward a physiological substrate, rather than the deficiency proposed above in the extended model, underlies the decreased angiogenicity of the N61D and N109D derivatives, which *do* compete with the native protein.

It is striking that the sites proposed for receptor binding in angiogenin correspond by homology to a region of RNase A that is in fact thought to make a substantial contribution to substrate-binding strength and specificity. Thus, X-ray crystallographic studies (Wodak et al., 1977) and molecular dynamics simulations (Brünger et al., 1985) show that Gln-69 and Asn-71 of RNase A—which lie within a loop analogous to that in angiogenin which contains residues 60–68—and Glu-111 (Glu-108 in angiogenin) form H bonds with the purine ring of substrates such as CpA. However, both the sequence (Strydom et al., 1985) and the calculated backbone structure of angiogenin (Palmer et al., 1986) differ dramatically from those of RNase A in each of these two regions: indeed angiogenin lacks the Cys-65–Cys-72 disulfide bond present in RNase and contains two fewer residues in each segment than does RNase. In functional terms, it is clear that neither the 60–68 loop nor the region around Asn-109 in angiogenin plays an important role in purine binding since dinucleotide cleavage is essentially unaffected by proteolytic removal of residues 61–67 (Hallahan et al., 1991), conversion of Asn-109 to isoaspartate (see above), or mutation of Glu-108 (T. P. Curran, personal communication). Furthermore, replacement of angiogenin residues 58–70 by the corresponding segment of RNase A enhances enzymatic activity by up to several hundred-fold[4] and alters specificity at the N′ position of NpN′ dinucleotide substrates (Harper & Vallee, 1989), while at the same time decreasing angiogenicity. Thus it seems that in both RNase and angiogenin this region has evolved to bind other molecules, but that the ligands recognized—RNA for RNase A and a cell-surface receptor for angiogenin—are different.

Finally, an obvious question raised by the present results is whether deamidation of angiogenin is a physiologically important process. Numerous proteins have been shown to undergo deamidation in vivo, including aldolase (Midelfort & Mehler, 1972), serine hydroxymethyltransferase (Artigues et al., 1990), and hemoglobin Providence (Moo-Penn et al., 1976). The rate of deamidation of angiogenin in vitro is relatively rapid, especially at Asn-61, and the products formed are biologically inactive. The primary, isoaspartyl products appear to be essentially inert but could be converted by carboxyl methyl transferases (Clarke, 1985), if accessible, to their aspartyl counterparts, which would be inhibitors of the native protein. Preliminary results, however, indicate that >90% of the immunologically detectable angiogenin in plasma coelutes with the unmodified protein during Mono S HPLC (K. A. Olson, personal communication). Thus, there appears to be no major accumulation of deamidation products in vivo. Whether this reflects a short lifetime for angiogenin in vivo or rapid specific clearance of the desamido products remains to be determined. If the Asp-61 and Asp-109 angiogenins are not in fact subject to accelerated clearance, these derivatives may be useful as angiogenin inhibitors for therapeutic use in limiting undesirable vascular proliferation, such as in growth of solid tumors.

[4] The angiogenin/RNase hybrid protein remains 10^2–10^4-fold less active than RNase A.

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REFERENCES

Ahern, T. J., & Klibanov, A. M. (1985) *Science 228*, 1280–1284.
Ahern, T. J., Casal, J. I., Petsko, G. A., & Klibanov, A. M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 675–679.
Artigues, A., Birkett, A., & Schirch, V. (1990) *J. Biol. Chem. 265*, 4853–4858.
Badet, J., Soncin, F., Guitton, J.-D., Lamare, O., Cartwright, T., & Barritault, D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8427–8431.
Becker, G. W., Tackitt, P. M., Bromer, W. W., Lefeber, D. S., & Riggin, R. M. (1988) *Biotechnol. Appl. Biochem. 10*, 326–337.
Bicknell, R., & Vallee, B. L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5961–5965.
Bicknell, R., & Vallee, B. L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1573–1577.
Bond, M. D., & Strydom, D. J. (1989) *Biochemistry 28*, 6110–6113.
Bond, M. D., & Vallee, B. L. (1990) *Biochem. Biophys. Res. Commun. 171*, 988–995.
Bornstein, P., & Balian, G. (1977) *Methods Enzymol. 47*, 132–145.
Brünger, A. T., Brooks, C. L., & Karplus, M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8458–8462.
Clarke, S. (1985) *Annu. Rev. Biochem. 54*, 479–506.
Clarke, S. (1987) *Int. J. Pept. Protein Res. 30*, 808–821.
Coughlin, S. R., Barr, P. J., Cousens, L. S., Fretto, L. J., & Williams, L. T. (1988) *J. Biol. Chem. 263*, 988–993.
Daumy, G. O., Wilder, C. L., Merenda, J. M., McColl, A. S., Geoghegan, K. F., & Otterness, I. G. (1991) *FEBS Lett. 278*, 98–102.
DiAugustine, R. P., Gibson, B. W., Aberth, W., Kelly, M., Ferrua, C. M., Tomooka, Y., Brown, C. F., & Walker, M. (1987) *Anal. Biochem. 165*, 420–429.
Di Donato, A., Galletti, P., & D'Alessio, G. (1986) *Biochemistry 25*, 8361–8368.
Fett, J. W., Strydom, D. J., Lobb, R. R., Alderman, E. M., Bethune, J. L., Riordan, J. F., & Vallee, B. L. (1985) *Biochemistry 24*, 5480–5486.
Gehrke, L., Jobling, S. A., Paik, L. S. K., McDonald, B., Rosenwasser, L. J., & Auron, P. E. (1990) *J. Biol. Chem. 265*, 5922–5925.
Geiger, T., & Clarke, S. (1987) *J. Biol. Chem. 262*, 785–794.
Gráf, L., Hajós, G., Patthy, A., & Cseh, G. (1973) *Horm. Metab. Res. 5*, 142–143.
Groskopf, W. R., Holleman, J. W., Margoliash, E., & Klotz, I. M. (1966) *Biochemistry 5*, 3783–3796.
Hallahan, T. W., Shapiro, R., & Vallee, B. L. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2222–2226.
Harper, J. W., & Vallee, B. L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7139–7143.
Harper, J. W., & Vallee, B. L. (1989) *Biochemistry 28*, 1875–1884.
Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene 77*, 51–59.
Hu, G.-F., Chang, S.-I., Riordan, J. F., & Vallee, B. L. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2227–2231.
Johnson, B. A., Langmack, E. L., & Aswad, D. W. (1987) *J. Biol. Chem. 262*, 12283–12287.
Ju, G., Labriola-Tompkins, E., Campen, C. A., Benjamin, W. R., Karas, J., Plocinski, J., Biondi, D., Kaffka, K. L., Kilian, P. L., Eisenberg, S. P., & Evans, R. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2658–2662.
Klagsbrun, M., & Baird, A. (1991) *Cell 67*, 229–231.
Knighton, D., Ausprunk, D., Tapper, D., & Folkman, J. (1977) *Br. J. Cancer 35*, 347–356.
Kossiakoff, A. A. (1988) *Science 240*, 191–194.
Kurachi, K., Davie, E. W., Strydom, D. J., Riordan, J. F., & Vallee, B. L. (1985) *Biochemistry 24*, 5494–5499.
Kurachi, K., Rybak, S. M., Fett, J. W., Shapiro, R., Strydom, D. J., Olson, K. A., Riordan, J. F., Davie, E. W., & Vallee, B. L. (1988) *Biochemistry 27*, 6557–6562.
Kurokawa, M., Doctrow, S. R., & Klagsbrun, M. (1989) *J. Biol. Chem. 264*, 7686–7691.
Liang, S.-M., Lee, N., Zoon, K. C., Manischewitz, J. F., Chollet, A., Liang, C.-M., & Quinnan, G. V. (1988) *J. Biol. Chem. 263*, 4768–4772.
Meinwald, Y. C., Stimson, E. R., & Scheraga, H. A. (1986) *Int. J. Pept. Protein Res. 28*, 79–84.
Midelfort, C. F., & Mehler, A. H. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1816–1819.
Moo-Penn, W. F., Jue, D. L., Bechtel, K. C., Johnson, M. H., Schmidt, R. M., McCurdy, P. R., Fox, J., Bonaventura, C., Sullivan, B., & Bonaventura, J. (1976) *J. Biol. Chem. 251*, 7557–7562.
Moore, F., & Riordan, J. F. (1990) *Biochemistry 29*, 228–233.
Moscatelli, D. (1987) *J. Cell. Physiol. 131*, 123–130.
Palmer, K. A., Scheraga, H. A., Riordan, J. F., & Vallee, B. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1965–1969.
Shapiro, R., & Vallee, B. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2238–2241.
Shapiro, R., & Vallee, B. L. (1989) *Biochemistry 28*, 7401–7408.
Shapiro, R., Fett, J. W., Strydom, D. J., & Vallee, B. L. (1986a) *Biochemistry 25*, 7255–7264.
Shapiro, R., Riordan, J. F., & Vallee, B. L. (1986b) *Biochemistry 25*, 3527–3532.
Shapiro, R., Strydom, D. J., Olson, K. A., & Vallee, B. L. (1987a) *Biochemistry 26*, 5141–5146.
Shapiro, R., Weremowicz, S., Riordan, J. F., & Vallee, B. L. (1987b) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8783–8787.
Shapiro, R., Harper, J. W., Fox, E. A., Jansen, H.-W., Hein, F., & Uhlmann, E. (1988) *Anal. Biochem. 175*, 450–461.
Shapiro, R., Fox, E. A., & Riordan, J. F. (1989) *Biochemistry 28*, 1726–1732.
Smyth, D. G., Stein, W. H., & Moore, S. (1962) *J. Biol. Chem. 237*, 1845–1850.
Stephenson, R. C., & Clarke, S. (1989) *J. Biol. Chem. 264*, 6164–6170.
Strydom, D. J., Fett, J. W., Lobb, R. R., Alderman, E. M., Bethune, J. L., Riordan, J. F., & Vallee, B. L. (1985) *Biochemistry 24*, 5486–5494.
Wodak, S. Y., Liu, M. Y., & Wyckoff, H. W. (1977) *J. Mol. Biol. 116*, 855–875.
Wright, H. T. (1991) *CRC Crit. Rev. Biochem. Mol. Biol. 26*, 1–52.
Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., & Ornitz, D. M. (1991) *Cell 64*, 841–848.

Registry No. Asn, 70-47-3; angiogenin, 97950-81-7; ribonuclease, 9001-99-4.