Role of Lysines in Human Angiogenin: Chemical Modification and Site-Directed Mutagenesis†

Robert Shapiro, Edward A. Fox, and James F. Riordan*
Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, Boston, Massachusetts 02115
Received September 7, 1988

ABSTRACT: The role of lysines in the ribonucleolytic and angiogenic activities of human angiogenin has been examined by chemical modification and site-directed mutagenesis. It was demonstrated previously [Shapiro , R., Weremowicz, S., Riordan, J. F., & Vallee, B. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8783-8787] that extensive treatment with lysine reagents markedly decreases the ribonucleolytic activity of angiogenin. In the present study, limited chemical modification with 1-fluoro-2,4-dinitrobenzene followed by C18 high-performance liquid chromatography yielded several (dinitrophenyl)angiogenin derivatives. The major derivative formed had slightly increased enzymatic activity compared with the unmodified protein. Tryptic peptide mapping demonstrated the site of modification to be Lys-50. A second derivative, modified at Lys-60, was 34% active. Analysis of a third derivative indicated that modification of Lys-82 did not decrease activity. Thus, Lys-50 and Lys-82 are unessential for enzymatic activity while Lys-60 may play a minor role. No pure derivative modified at Lys-40, corresponding to the active-site residue Lys-41 of the homologous protein ribonuclease A, could be obtained by chemical procedures. Therefore, we employed oligonucleotide-directed mutagenesis to replace this lysine with glutamine or arginine. The Gln-40 derivative had <0.05% enzymatic activity compared with the unmodified protein and substantially reduced angiogenic activity when examined with the chick embryo chorioallantoic membrane assay. These results suggest that the angiogenic activity of the protein is dependent on an intact enzymatic active site. The Arg-40 derivative had 2.2% ribonucleolytic activity compared with unmodified angiogenin. The effects of reductive methylation of this derivative indicate that no lysines other than Lys-40 are critical. Examination of the interaction of Arg-40 angiogenin with human placental ribonuclease inhibitor, an extremely tight binding inhibitor of angiogenin, reveals a 100-fold increase in K_i compared with the Lys-40 protein, due entirely to an increase in the rate of dissociation.

Human angiogenin is a 14 124-dalton protein originally isolated on the basis of its capacity to induce neovascularization on the chick embryo chorioallantoic membrane (CAM)[1] (Fett et al., 1985). Determination of the amino acid sequence of this protein (Strydom et al., 1985; Kurachi et al., 1985) revealed 35% identity with pancreatic RNase, including virtually all of the residues thought to be important for activity of that enzyme. It was subsequently found that angiogenin also has ribonucleolytic activity, although its action differs distinctly from that of pancreatic RNase (Shapiro et al., 1986; St. Clair et al., 1987). A preliminary three-dimensional structure of angiogenin, computed by energy minimization procedures utilizing the backbone structure of bovine pancreatic RNase A (Palmer et al., 1986), suggests that in angiogenin the overall structural motif of RNase is conserved.

Chemical modification has indicated that angiogenin, like RNase A, contains essential histidine and lysine residues (Shapiro et al., 1986, 1987b). Treatment with bromoacetate at pH 5.5 markedly decreases both ribonucleolytic and angiogenic activity through specific reaction with His-13 and His-114 (Shapiro et al., 1986, 1988b). Similarly, lysine reagents abolish ribonucleolytic activity, but the reactions are not specific (Shapiro et al., 1987b), and it has not been possible to correlate inactivation with derivatization of a particular residue. The homology with RNase A implicates Lys-40 as a residue that is critical for enzymatic activity.

In the present study, we have further explored the role of lysine residues in angiogenin, employing both chemical modification and site-directed mutagenesis. Though chemical derivatization of lysines at positions 50, 60, and 82 demonstrates them to be nonessential, mutagenesis of Lys-40 reveals it to be critical for both the ribonucleolytic and angiogenic activities of angiogenin.

Experimental Procedures

Angiogenin was obtained from human plasma (Shapiro et al., 1987a) or from recombinant expression systems in mammalian cells (Kurachi et al., 1988) or *Escherichia coli* (Shapiro et al., 1988a). Stock solutions of the protein were quantitated by amino acid analysis. Bovine pancreatic RNase A was purchased from Cooper Biomedical and was quantitated by using a molar absorptivity at 278 nm of 9800 M^{-1} cm^{-1} (Sela & Anfinsen, 1957). Human placental ribonuclease inhibitor (PRI) was isolated by the method of Blackburn (1979). Concentrations of PRI solutions were determined by inhibition of RNase A activity toward CpG (see below), using a four-point titration plot. Such a plot is linear under the conditions

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

* Address correspondence to this author at the Center for Biochemical and Biophysical Sciences and Medicine, Seeley G. Mudd Building, 250 Longwood Ave., Boston, MA 02115.

[1] Abbreviations: CAM, chorioallantoic membrane; RNase, ribonuclease; PRI, human placental RNase inhibitor; CpG, cytidylyl(3′→5′)guanosine; FDNB, 1-fluoro-2,4-dinitrobenzene; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; C18, octadecylsilane; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; C3, propylsilane; K40Q-angiogenin, Met-(−1) angiogenin in which Lys-40 is replaced by glutamine; K40R-angiogenin, Met-(−1) angiogenin in which Lys-40 is replaced by arginine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

0006-2960/89/0428-1726$01.50/0

employed, i.e., [PRI] $\gg K_i$. The expression vector pAng2, containing a synthetic angiogenin gene, was prepared as described (Shapiro et al., 1988a). It contains a modified *E. coli trp* promoter, an rrn^B transcription termination sequence, and an ampicillin resistance marker for selection.

Chemical Modification. Reactions of angiogenin (10–20 μM) with 1-fluoro-2,4-dinitrobenzene (FDNB) were performed in 50 mM Hepes, pH 8.0, at 26 °C in the dark. (Dinitrophenyl)angiogenin derivatives B and E (Figure 1) used for peptide mapping were obtained by incubating 125 μg of angiogenin with 1.5 mM FDNB for 35 min. Derivative D for peptide mapping and all derivatives employed for measurements of enzymatic activity were obtained by incubating 480 μg of angiogenin with 3.0 mM FDNB for 45 min. Modified angiogenin derivatives were isolated by C18 HPLC on a Synchropak RP-P column (250 × 4.6 mm; Synchrom, Inc.). Elution was achieved at a flow rate of 1.0 mL/min with a gradient of 30–60% solvent B in 135 min, where solvent A was 0.1% TFA in water (v/v) and solvent B was 0.08% TFA in a 3:2:2 mixture of 2-propanol/acetonitrile/water. One-minute fractions were collected. Reductive methylation of angiogenin was performed with 2 mM formaldehyde and 10 mM sodium cyanoborohydride in 50 mM Mes, pH 6.0 (Shapiro et al., 1987b).

Oligonucleotide-Directed Mutagenesis. Genes encoding angiogenin with a glutamine or arginine residue replacing Lys-40 were obtained by using the Bio-Rad Muta-Gene mutagenesis kit as described (Shapiro et al., 1988a). The mutagenic oligonucleotides were pAGTCCGTGCCAAGATATCAAC (Gln-40) and pGTCCGTGCAGAGATATCAAC (Arg-40). Identification of plaques containing DNA sequences coding for the desired angiogenin derivatives was accomplished by chain termination DNA sequencing (Sanger et al., 1977; Tabor & Richardson, 1987) using a Sequenase kit from United States Biochemicals. Genes were transferred from M13mp18 into the expression vector pAng2 as described (Shapiro et al., 1988a).

Expression and Isolation of Angiogenin Derivatives. Expression and purification of recombinant angiogenins[2] and immunoblotting were performed as described previously (Shapiro et al., 1988a). Material eluting from the C18 HPLC column was dialyzed against water prior to being tested for biological and enzymatic activity.

Structural Characterization. Amino acid analyses (Picotag method; Waters Associates) and digestions with HPLC-purified trypsin were performed as described (Strydom et al., 1985; Shapiro et al., 1988a). Tryptic peptides were purified by reversed-phase HPLC on a Beckman Ultrapore C3 column (FDNB-modified angiogenin, peaks B and E) or a Beckman Ultrasphere C18 column (FDNB-modified angiogenin, peak D, and mutant angiogenins). Eluates from FDNB-modified angiogenin were monitored simultaneously at 206 or 214 nm and at 436 nm. The solvents were as described above. With the C3 column, a linear gradient from 5% to 95% solvent B in 3 h was employed at a flow rate of 1 mL/min. With the C18 column, a linear gradient from 0% to 50% solvent B in 140 min was employed at 0.8 mL/min. One-minute fractions were collected.

Enzymatic Assays. Activity toward yeast tRNA (type X, Sigma Chemical Co.) was determined by measuring formation of perchloric acid soluble fragments as described (Shapiro et al., 1987b). For (dinitrophenyl)angiogenin derivatives, the incubation time at 37 °C was 4 h, and the enzyme concentration was 0.23 μM. For K40Q- and K40R-angiogenins, the reaction times were 4 and 5 h, respectively, and enzyme concentrations of 10.6 μM (K40Q) and up to 4.6 μM (K40R) were employed. Incubations with reductively methylated K40R-angiogenin were 8 h, and the enzyme concentration was 3.9 μM.

Biological Assays. Angiogenic activity was assessed by the chick embryo chorioallantoic membrane method (Knighton et al., 1977; Fett et al., 1985). The number of eggs employed in any individual set of assays at a given concentration ranged from 6 to 14.

Circular Dichroic Spectra. Spectra were recorded at 25 °C on a Cary 60 spectropolarimeter. Angiogenin derivatives were ~1.5 μM in 1 mM Tris, pH 8.0. Molar ellipticity was calculated as a function of wavelength from the observed spectrum corrected for the spectrum of the buffer alone in the identical optical cell.

Interaction of Angiogenin Derivatives with Human Placental Ribonuclease Inhibitor. (A) Association Rate. The apparent second-order rate constants for association of PRI with angiogenin and K40R-angiogenin were determined by examining their competition with RNase A for PRI (Shapiro & Vallee, 1987; Lee et al., 1988a). RNase A (5 nM) was mixed with 0.8–1.4 equiv of angiogenin at 25 °C in 0.1 M Mes, pH 6.0, containing 0.1 M NaCl and 1 mM EDTA. PRI was then added to a final concentration of 5 nM. After 15 s, the activity of free RNase A was then determined by adding CpG (final concentration 100 μM) and continuously monitoring the decrease in absorbance at 286 nm on a Varian Model 219 spectrophotometer. Angiogenin does not cleave CpG at a detectable rate (Shapiro et al., 1986). The apparent second-order rate constant of association was then calculated by using the equation:

$$k_{assoc,A} = k_{assoc,R} \ln ([A]_T/[A]_F)/\ln ([R]_T/[R]_F)$$

where $k_{assoc,R}$ is the apparent second-order rate constant for association of PRI with RNase A, $[A]_T$ and $[A]_F$ are the total and free angiogenin concentrations, respectively, and $[R]_T$ and $[R]_F$ are the total and free RNase A concentrations, respectively (Lee et al., 1988a).[3] It has been shown previously that $k_{assoc,R}$ under the conditions employed is 3.4×10^8 M^{-1} s^{-1} (Lee et al., 1988a).

(B) Dissociation Rate. The rate constant for dissociation of K40R-angiogenin from its complex with PRI was determined by first forming the complex, then adding a vast excess of RNase A as a scavenger for free PRI, and finally determining the amounts of free angiogenin derivative at various times by cation-exchange HPLC (Lee et al., 1988b). Final mixtures contained 0.7 μM angiogenin, 1.0 μM PRI, and 175 μM RNase A in 0.1 M Mes, pH 6.0, with 0.1 M NaCl, 1 mM EDTA, and 0.12 mM DTT at 25 °C. RNase was added after preincubation of the remaining components at 25 °C for 20 min. At various times, 140-μL aliquots were chromatographed on a Synchropak CM300 HPLC column (250 × 4.1 mm;

[2] Angiogenin produced in *Escherichia coli* differs from the naturally occurring protein only with respect to its amino terminus: Met-(−1) and <Glu-1, respectively. This difference does not influence ribonucleolytic or angiogenic activity (Shapiro et al., 1988a). For simplicity, bacterially produced Met-(−1) angiogenin is referred to as "angiogenin".

[3] It has been shown previously (Lee et al., 1988a) that the association of angiogenin and PRI follows a two-step mechanism:

$$E + I \underset{}{\overset{K_1(fast)}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^*$$

where E and I represent angiogenin and PRI, respectively. The rate of association approximates k_2/K_1 when both [E] and [I] are well below K_1. In the assays described, these conditions are fulfilled for unmodified angiogenin, since K_1 = 530 nM, [E] = 5 nM, and [I] < 7 nM.

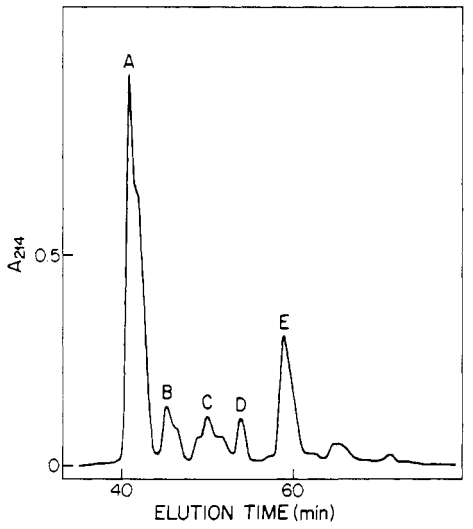

FIGURE 1: Chromatography of FDNB-modified angiogenin on a C18 HPLC column. Angiogenin (125 μg) was treated with 1.5 mM reagent for 35 min at 25 °C in 1.0 mL of 50 mM Hepes, pH 8.0. Elution was achieved at 1 mL/min with a 135-min linear gradient from 30% to 60% solvent B (see Experimental Procedures).

Synchrom, Inc.) using a 15-min linear gradient from 0.2 to 0.6 M NaCl in 20 mM sodium phosphate, pH 7.0, at a flow rate of 1 mL/min. A Waters Associates HPLC system equipped with a 214-nm detector and a Model 730 data module was employed. Free angiogenin was well resolved from the other components and was quantitated by peak area. A control sample lacking PRI, assayed at the end of the experiment, showed that there was no significant loss of angiogenin during the course of the incubation.

RESULTS

Isolation of Dinitrophenyl Derivatives of Angiogenin. Treatment of angiogenin with 50 mM FDNB at pH 8.0 for 1 h at 27 °C causes 80% loss of its ribonucleolytic activity (Shapiro et al., 1987b). During this time, 3.8 of the 7 lysines of angiogenin are modified. In order to obtain angiogenin derivatives that have been dinitrophenylated at a single site, the protein was treated under milder conditions (1.5 mM reagent, 35 min) expected to modify <0.5 lysine and then subjected to C18 HPLC. Five major peaks were observed (Figure 1). Peak A represents unmodified angiogenin, as judged by both elution time and amino acid composition. Peaks B, C, D, and E show losses of 1.05, 0.95, 0.69, and 0.97 lysine residues, respectively.

Ribonucleolytic Activity of (Dinitrophenyl)angiogenin Derivatives. HPLC fractions containing material eluting in peaks A–E were lyophilized, reconstituted in water, and assayed for activity toward tRNA. Only the derivative in peak B had decreased activity, 34% compared with native angiogenin. Material in peak A was 100% active, again consistent with it being unmodified angiogenin. Derivatives in peaks C, D, and E were all somewhat more active than native angiogenin, by 15%, 50%, and 14%, respectively.

Structural Characterization of (Dinitrophenyl)angiogenin Derivatives. The peak B, D, and E derivatives were digested with trypsin, and the resulting peptides were separated by C3 or C18 HPLC (see Experimental Procedures). Peak C appeared to be heterogeneous and was not analyzed further. Peptides containing (dinitrophenyl)lysine were identified by monitoring HPLC eluates at 436 nm. The peptide map for peak B contained one such peptide, which by amino acid analysis (Table I) was identified as angiogenin sequences 55–66 and 102–121, normally in peptides T4a and T11 [see Strydom et al. (1985) for a description of angiogenin tryptic peptides]. Only modification of Lys-60 would produce this result.

Table I: Amino Acid Compositions of the Major 436-nm-Absorbing Peptides from (Dinitrophenyl)angiogenin Derivatives[a]

amino acid	major 436-nm-absorbing peptide from peak B[b]	peak D[c]	peak E[d]
Asx	5.50 (6)	5.02 (5)	3.45 (3)
Glx	4.16 (3)	3.57 (3)	0.49
Ser	2.80 (1)	4.49 (5)	0.68
Gly	4.06 (2)	4.86 (4)	1.63 (1)
His	1.75 (2)	1.92 (2)	0.88 (1)
Arg	1.73 (2)	3.80 (4)	0.90 (1)
Thr	0.64	3.55 (4)	1.14 (1)
Ala	3.10 (2)	1.00	0.43
Pro	2.35 (2)	3.82 (4)	0.34
Tyr		1.72 (2)	
Val	3.62 (4)	1.53 (1)	0.23
Met		0.96 (1)	
Ile	2.14 (2)	2.52 (3)	1.99 (2)
Leu	2.68 (2)	2.49 (2)	0.35
Phe	1.17 (1)	2.03 (2)	1.08 (1)
Lys	0.91 (1)	2.58 (3)	0.17 (1)
pmol analyzed	13	34	30

[a] Cystine and tryptophan contents were not determined. [b] Numbers in parentheses represent composition of the disulfide-linked angiogenin sequences 55–66 + 102–121. [c] Numbers in parentheses represent composition of angiogenin sequences 22–31, 34–51, and 74–95 with two disulfide bonds linking half-cystinyl residues 26–81 and 39–92. [d] Numbers in parentheses represent composition of angiogenin sequence 41–51.

The peptide map for peak D was devoid of T10 and had substantially decreased amounts of T8 and T9. Amino acid analysis of the major 436-nm-absorbing peptide (Table I) indicates that it contains equimolar amounts of angiogenin sequences 22–31, 34–51, and 74–95, normally found in peptides T8, T9, and T10. The results are consistent with modification at either Lys-40 or Lys-82, with trypsin failing to cleave at either site. This peptide was not characterized further due to lack of material.

The peptide map for the major derivative formed, peak E, lacked peptide T8 and contained a single major 436-nm-absorbing peptide. Amino acid analysis of this peptide (Table I) identified it as T8, containing the angiogenin sequence 41–51, except for the virtual absence of lysine. Thus, Lys-50 is the site of modification.

Preparation of Angiogenin Mutants. Genes encoding K40Q- and K40R-angiogenin were produced by oligonucleotide-directed mutagenesis in M13 (see Experimental Procedures). The DNA for each mutant was sequenced in its entirety in order to rule out any additional mutations. Mutant proteins were expressed in *E. coli* and purified to homogeneity as described previously (Shapiro et al., 1988a). There were no marked changes in chromatographic behavior during cation-exchange or C18 HPLC of the mutagenic derivatives compared with angiogenin. All preparations were at least 99% pure as judged by SDS–PAGE (not shown). Immunoblotting of *E. coli* cell extracts revealed levels of expression of both mutants 2–5 times higher than for the Lys-40 protein, and yields of up to 5 mg/L culture were obtained.

Structural Characterization of Angiogenin Mutants. Amino acid compositions of both K40Q and K40R mutants (Table II) were in excellent agreement with their proposed structures. As with the unmodified angiogenin, the presence of two methionines indicates that both proteins contain a Met-(−1) residue. Tryptic peptide mapping (Figure 2) was performed in order to determine the pairing of disulfide bonds and to ensure that no unexpected changes in primary structure had

Table II: Amino Acid Compositions of Angiogenin and Lys-40 Mutants Produced in *E. coli*[a]

amino acid	mutants		
	angiogenin	K40Q	K40R
Asx	15.3 (15)	15.3	15.3
Glx	10.0 (10)	10.9	10.1
Ser	8.4 (9)	8.5	8.0
Gly	8.0 (8)	7.9	8.0
His	5.9 (6)	6.0	5.8
Arg	13.0 (13)	12.9	14.2
Thr	6.7 (7)	6.7	6.8
Ala	5.1 (5)	5.1	5.1
Pro	8.1 (8)	8.1	8.0
Tyr	3.9 (4)	4.0	3.8
Val	4.1 (5)	4.1	4.5
Met	2.1 (2)	2.2	2.0
Ile	6.5 (7)	6.6	6.9
Leu	5.9 (6)	5.8	6.1
Phe	4.9 (5)	4.9	4.9
Lys	7.0 (7)	6.0	5.7
Cys	5.7 (6)	5.7	5.6

[a] Analyses were performed in duplicate as described (Strydom et al., 1985). In parentheses are the numbers of residues expected on the basis of the amino acid sequence of angiogenin.

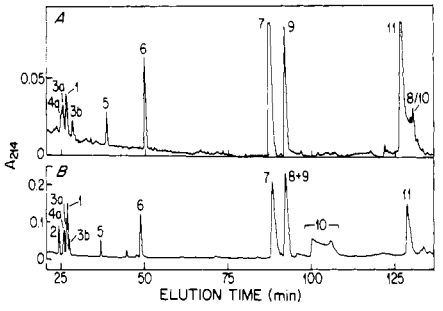

FIGURE 2: Chromatography of tryptic digests of 2.2 nmol of K40Q-angiogenin (panel A) and 3.4 nmol of K40R-angiogenin (panel B) on a C18 HPLC column.

occurred. The peptide maps of both proteins were entirely consistent with the expected structures. With native angiogenin, peptide T10 contains the disulfide-linked sequences 34–40 and 83–95. With K40Q-angiogenin, peptides T8 (amino acids 41–51) and T10 are absent, and a new peptide denoted T8/10 elutes immediately after T11. The composition of this peptide (Table III) indicates the presence of the angiogenin sequence 83–95 and the modified sequence 34–51 expected for this mutation. Analyses of all other peptides were in good agreement with those of the unmodified protein. Compositions of these peptides demonstrate the formation of all three disulfides as in native angiogenin and account for all but four residues of the protein (Arg-32, Arg-33, Arg-122, and Pro-123).

The peptide map of the K40R mutant is essentially indistinguishable from that of the unmodified protein. In peptide T10 (Table III), an arginine replaces a lysine, and there are no other changes. The compositions of the remaining peptides are indistinguishable from those of unmodified angiogenin and account for the entire molecule, except for Arg-32 and Arg-33. The composition of peptides T9, T10, and T11 again confirm that the disulfide bonds have formed correctly.

Circular Dichroic Spectra. The circular dichroic spectra of unmodified and K40Q- and K40R-angiogenins measured from 205 to 270 nm are essentially identical (Figure 3) and demonstrate that the mutant angiogenins had not undergone any extensive changes in secondary structure.

Enzymatic Activity of Angiogenin Mutants. The ribonucleolytic activity of mutant angiogenins was quantitated by measuring the formation of perchloric acid soluble fragments from yeast tRNA. The K40Q mutant is essentially inactive, allowing an upper limit to be set at 0.05% activity compared with the unmodified enzyme. The K40R mutant, in contrast, is 2.2% as active as angiogenin itself.

Biological Activity of K40Q-Angiogenin. The angiogenic activity of the K40Q mutant was examined extensively at the 1-, 5-, and 10-ng level using the CAM assay (Table IV). These assays reveal that the activity of the mutant protein is

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

amino acid	T8/10 K40Q	T10 K40R
Asx	3.81 (3)	0.26
Glx	2.59 (2)	1.23 (1)
Ser	1.84 (2)	1.95 (2)
Gly	3.65 (4)	3.25 (3)
His	1.80 (2)	1.00 (1)
Arg	1.76 (2)	2.03 (2)
Thr	1.32 (2)	1.04 (1)
Ala	0.87	0.25
Pro	3.43 (4)	3.73 (4)
Tyr	0.70 (1)	0.81 (1)
Val	1.31	
Met		
Ile	2.09 (2)	
Leu	2.22 (2)	2.04 (2)
Phe	1.06 (1)	
Lys	1.05 (1)	0.29
pmol analyzed	24	60

[a] Cystine and tryptophan contents were not determined. Numbers in parentheses represent compositions of the mutants expected for disulfide-linked amino acids 34–51 + 83–95 (K40Q) or 34–40 + 83–95 (K40R). Values for some amino acids in the K40Q peptide are higher than expected due to contamination by peptide T11, which elutes immediately preceding it.

Table IV: Angiogenic Activity of Angiogenin and K40Q-Angiogenin[a]

sample	dose (ng)	% positives (total number of eggs)
angiogenin	10	60 (62)
	5	58 (69)
	1	49 (61)
K40Q-angiogenin	10	27 (64)
	5	27 (66)
	1	21 (53)

[a] The CAM assay was employed (Knighton et al., 1977; Fett et al., 1985). Between 6 and 14 eggs were used for each set of assays at a given concentration. Data obtained with two different preparations of both angiogenin and K40Q-angiogenin did not differ significantly and were combined. Control samples containing only water assayed simultaneously on a total of 56 eggs were 18% positive (range 10–33%).

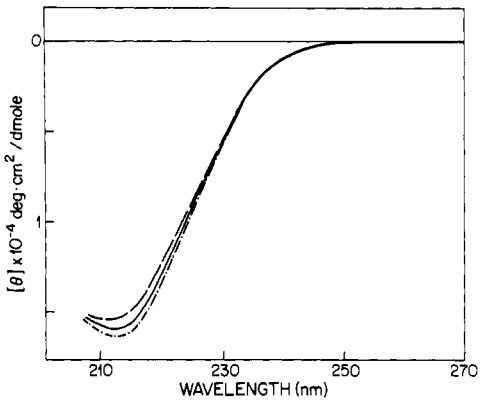

FIGURE 3: Circular dichroic spectra of angiogenin (—), K40Q-angiogenin (-·-), and K40R-angiogenin (--) in 1 mM Tris, pH 8.0. Negative ellipticity is plotted vs wavelength.

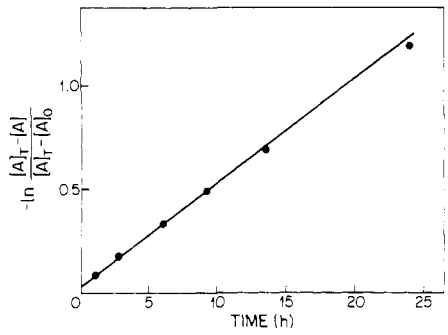

FIGURE 4: Dissociation of K40R-angiogenin–PRI complex. K40R-Angiogenin was preincubated with 1.5 equiv of PRI for 20 min at 25 °C. Release of free angiogenin was measured by cation-exchange HPLC after addition of 250 equiv of RNase A. Data are plotted as $-\ln [([A]_T - [A])/([A]_T - [A]_0)]$ vs time, where $[A]_T$ is the total angiogenin concentration, [A] is the concentration of free angiogenin, and $[A]_0$ is the free angiogenin concentration several minutes after addition of scavenger.

substantially reduced; even at 10 ng/egg, the percentage of positive responses is considerably lower than that observed with the unmodified protein at 1 ng/egg.

Reductive Methylation of K40R-Angiogenin. As noted above, the K40R mutant has detectable activity toward tRNA. In order to determine whether this activity is affected by lysine modification, the mutant protein was reductively methylated with formaldehyde and sodium cyanoborohydride. Under conditions where the enzymatic activity of native angiogenin decreases by 97.7%, the activity of K40R-angiogenin decreases only by 59%, i.e., from 2.2% to 1.0%.

Interaction of K40R-Angiogenin with Human Placental RNase Inhibitor. Angiogenin binds extremely tightly to human placental RNase inhibitor [$K_i = 7 \times 10^{-16}$ M (Lee et al., 1988b)], a 51 000-dalton cytoplasmic protein (Blackburn et al., 1977). An examination of the interaction of K40R-angiogenin with PRI reveals an apparent second-order rate constant of association, measured by using a competition assay (see Experimental Procedures), of $(3.7 \pm 0.4) \times 10^8$ M^{-1} s^{-1} at pH 6, 25 °C, essentially the same as $(4.0 \pm 0.3) \times 10^8$ M^{-1} s^{-1} for angiogenin. In both cases, the Met-(−1) species was employed, and these association rates are ~2-fold higher than for the naturally occurring <Glu-1 protein.

The rate of dissociation of K40R-angiogenin from its complex with PRI was examined by first forming the complex and subsequently using HPLC to monitor the release of the free angiogenin derivative after addition of a 250-fold molar excess of RNase as scavenger for free PRI. A small amount of free K40R-angiogenin, 10.3% of the total, was released within several minutes after addition of scavenger, with the remainder released more slowly. Analysis of the slow dissociation as a first-order process (Figure 4) gives a rate constant of 1.4×10^{-5} s^{-1}, corresponding to a half-life of 14 h. After 48 h, ~98% of the angiogenin derivative had been released. This rate is 100-fold faster than for native angiogenin (Lee et al., 1988b). The rate of dissociation with Met-(−1) angiogenin is indistinguishable from that of the natural <Glu-1 protein.

Studies on the interaction of K40Q-angiogenin with PRI are reported elsewhere (F. Lee et al., submitted for publication; see Discussion).

Discussion

The homology between angiogenin and RNase A identifies Lys-40 of angiogenin as a likely candidate for a functionally critical residue. It has long been known that RNase A is inactivated by lysine reagents and in several cases this effect can be attributed unequivocally to modification of the corresponding residue, Lys-41. FDNB (Hirs et al., 1965), bromoacetate at alkaline pH (Heinrikson, 1966), pyridoxal phosphate (Raetz & Auld, 1972), 2-carboxy-4,6-dinitrochlorobenzene (Bello et al., 1979), and others react fairly specifically with this residue, and inactive RNase derivatives have been isolated that are modified exclusively at this site. X-ray crystallographic and neutron diffraction studies (Richards & Wyckoff, 1973; Borkakoti et al., 1983; Wlodawer et al., 1983, 1988; Campbell & Petsko, 1988) clearly place Lys-41 at the active site and suggest that it may stabilize the pentacovalent intermediate or transition state (Roberts et al., 1969; Richards & Wyckoff, 1971; Holmes et al., 1978; Alber et al., 1983) or facilitate deprotonation of the substrate 2′-hydroxyl group (Wlodawer et al., 1983). However, despite a vast accumulation of chemical and physical data, an essential role for Lys-41 has not yet been rigorously established. Chemical modifications might decrease activity due to steric perturbations caused by the additional substituent rather than loss of the functional group in question, and published crystal structures have thus far examined only the free enzyme and its complexes with various inhibitors. No mutagenesis studies have been reported, and only a preliminary, qualitative description of RNase crystal structure during catalysis (Alber et al., 1983) is available.

A variety of reagents specific for modifying lysine residues also markedly decrease the ribonucleolytic activity of angiogenin (Shapiro et al., 1987b). These include diethyl pyrocarbonate, acetic anhydride, citraconic anhydride, FDNB, and formaldehyde plus sodium cyanoborohydride. Identification of the critical lysine residue(s) by chemical modification techniques, however, has been complicated by the lack of specificity of these reagents. Thus, under conditions where angiogenin is ~80% inactivated by FDNB or reductive methylation, 3.8 or 6.3 of the 7 lysines are modified, respectively. Poly(G), an inhibitor that protects almost completely against the effects of reductive methylation (Shapiro et al., 1987b), also prevents modification of about five lysines (unpublished results), precluding the use of this inhibitor for identifying a specific essential residue.

The present study has utilized both chemical modification and mutagenic approaches to more clearly define the role of lysines in angiogenin. Limited chemical modification with FDNB followed by C18 HPLC yielded two angiogenin derivatives, each dinitrophenylated at a single lysine. The major species formed was modified at Lys-50 and had slightly increased ribonucleolytic activity compared with that of the unmodified enzyme. Thus, this residue is clearly not essential for activity. The enzymatic activity of another derivative, modified at Lys-60, decreased somewhat to 34% of that of angiogenin. This decrease could reflect either a minor functional role for this residue or more general effects due to the bulky dinitrophenyl group added. The corresponding residue, Lys-61, in the RNase crystal structure is located fairly distant from the active site (Richards & Wyckoff, 1973), but it could be involved in secondary interactions with large substrates. Indeed, Blackburn and Gavilanes (1982) have reported that poly(A) completely protects Lys-61 against amidination.

A third (dinitrophenyl)angiogenin derivative was also isolated, but in this case modification at Lys-40, Lys-82, or at a mixture of the two sites could not be differentiated. Mutagenesis experiments and analogy to RNase strongly suggest that modification at Lys-40 would produce an inactive protein. Since the third dinitrophenyl derivative is in fact more active than angiogenin itself, it can be deduced that some of the material in peak D (Figure 1) is modified at Lys-82 and that

this derivative is at least as active as the unmodified enzyme. Therefore, Lys-82 would also seem to be nonessential for enzymatic activity.

Since specific modification of Lys-40 and concomitant definition of its function by chemical procedures had not proved feasible, the role of this residue in angiogenin was examined by replacing it with glutamine and arginine, changes that are relatively conservative. Glutamine, an uncharged hydrophilic residue of size similar to lysine, decreased ribonucleolytic activity at least 2000-fold, the limit of detection in the assay employed. This clearly indicates a critical role for Lys-40. However, the activity of the derivative in which arginine replaces Lys-40 was reduced only 45-fold. This suggests that whatever role this lysine may play it can be partially fulfilled by another positively charged residue of similar size. These findings may or may not pertain to RNase A.

Since K40R-angiogenin retains measurable enzymatic activity, it was possible to examine the role of the other lysines in this derivative by reductive methylation. Only a modest decrease in activity of the mutant protein was observed under conditions where angiogenin itself loses 98% activity. Thus, it would appear that methylation of Lys-40 is responsible for the almost complete inactivation of angiogenin. The partial loss of activity of the K40R derivative may reflect less critical roles for one or more of the remaining lysines, perhaps Lys-60 (see above).

The molecular mechanism by which angiogenin induces neovascularization is unknown. A critical question in this regard is whether the biological action of the protein is dependent on its ribonucleolytic activity. Carboxymethylation of active-site histidines markedly decreases both activities (Shapiro et al., 1986), suggesting that they are indeed related. This is further supported by the finding that PRI abolishes both activities (Shapiro & Vallee, 1987). In the latter case, in particular, the interpretation must be guarded since the physical properties of the complex, e.g., charge and rate of diffusion, differ from those of free angiogenin. Moreover, the inhibitor has 3.6 times the molecular mass of angiogenin and may cover extensive areas beyond that of the enzymatic active site. Concerning the second point, however, a recent study of the interaction of PRI with angiogenin derivatives (F. Lee et al., submitted for publication) suggests that the contact region may be considerably smaller than that proposed for the PRI·RNase complex (Blackburn & Moore, 1982).

With the availability of mutants of angiogenin, the relationship between its enzymatic and biological activities can now be established more rigorously. It has been demonstrated recently (Harper & Vallee, 1988) that replacement of Asp-116 by histidine increases both activities dramatically. The present work shows that replacement of Lys-40 by glutamine markedly reduces both activities. These findings strongly suggest that the angiogenic activity of the protein is dependent on an intact enzymatic active site. At present, it cannot be differentiated whether or not this action requires that the site be catalytically competent or only that it retains the capacity to bind the appropriate molecule. It should also be recognized that additional regions of angiogenin outside the active site may be critical for biological activity, e.g., for receptor binding. Attempts to delineate any such regions are now in progress.

In addition to their effects on ribonucleolytic and angiogenic activity, replacements of Lys-40 weaken binding to PRI considerably. The rates of association and dissociation of K40Q-angiogenin and PRI are reported elsewhere (F. Lee et al., submitted for publication) as part of a more detailed examination of the effects of various modifications of angiogenin on its interaction with the inhibitor. This substitution, in particular, decreases the association rate 3-fold and increases the dissociation rate 440-fold. Thus, the overall effect on K_i is an increase of 1300-fold. Even the seemingly conservative Lys → Arg substitution examined here produces a 100-fold increase in K_i, due entirely to an effect on the rate of dissociation. This suggests that the interaction of Lys-40 with PRI is highly specific and can be accommodated only partially by an arginine.

Whenever a mutant protein is inactive, concerns about the overall integrity of its three-dimensional structure must be addressed. In the present case, several pieces of evidence suggest that the Gln-40 and Lys-40 angiogenins do not differ significantly except at the site of replacement. First, peptide mapping (Figure 2) demonstrates correct formation of the three disulfide bonds, already placing considerable constraints on the general shape of the molecule. Second, the C18 HPLC elution time of the mutant differs only slightly from that of unmodified angiogenin. Elution time in the system employed is an extremely sensitive structural indicator. Thus, angiogenin with an internal cleavage at Lys-60 elutes several minutes later than the intact protein (Harper & Vallee, 1988b), and partial reduction of disulfide bonds causes significantly greater retention (unpublished results). Third, the circular dichroic spectra of the mutant and Lys-40 angiogenins are indistinguishable (Figure 3), implying that there are no major changes in secondary structure. Finally, and perhaps most convincingly, the K40Q derivative still binds to PRI with a K_i value of 9.2×10^{-13} M (F. Lee et al., submitted for publication). A 1300-fold increase in K_i for this derivative compared with native angiogenin is consistent with expectations for the loss of a single important ionic interaction ($\Delta G \sim 4$ kcal/mol).

Acknowledgments

We thank Dr. Bert L. Vallee for continued advice and support, Dr. Daniel J. Strydom for peptide mapping, and George Cohen and Nazik Sarkissian for excellent technical assistance.

Registry No. RNase, 9001-99-4; PRI, 39369-21-6; L-Lys, 56-87-1; L-Gln, 56-85-9; L-Arg, 74-79-3.

References

Alber, T., Gilbert, W. A., Ponzi, D. R., & Petsko, G. A. (1983) *Ciba Symp. 93*, 4–24.

Bello, J., Iijima, H., & Kartha, G. (1979) *Int. J. Pept. Protein Res. 14*, 199–212.

Blackburn, P. (1979) *J. Biol. Chem. 254*, 12484–12487.

Blackburn, P., & Gavilanes, J. G. (1982) *J. Biol. Chem. 257*, 316–321.

Blackburn, P., & Moore, S. (1982) *Enzymes (3rd Ed.) 15*, 317–433.

Blackburn, P., Wilson, G., & Moore, S. (1977) *J. Biol. Chem. 252*, 5904–5910.

Borkakoti, N., Palmer, R. A., Haneef, I., & Moss, D. S. (1983) *J. Mol. Biol. 169*, 743–755.

Campbell, R. L., & Petsko, G. A. (1987) *Biochemistry 26*, 8579–8584.

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.

Harper, J. W., & Vallee, B. L. (1988a) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7139–7143.

Harper, J. W., & Vallee, B. L. (1988b) *J. Protein Chem. 7*, 355–363.

Heinrikson, R. L. (1966) *J. Biol. Chem. 241*, 1393–1405.

Hirs, C. H. W., Halmann, M., & Kycia, J. H. (1965) *Arch. Biochem. Biophys. 111*, 209-222.
Holmes, R. R., Deiters, J. A., & Gallucci, J. C. (1978) *J. Am. Chem. Soc. 100*, 7393-7402.
Knighton, D., Ausprunk, D., Tapper, D., & Folkman, J. (1977) *Br. J. Cancer 35*, 347-356.
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. F., Shapiro, R., Strydom, D. J., Olson, K. A., Riordan, J. F., Davie, E. W., & Vallee, B. L. (1988) *Biochemistry 27*, 6557-6562.
Lee, F. S., Auld, D. S., & Vallee, B. L. (1988a) *Biochemistry* (in press).
Lee, F. S., Shapiro, R., & Vallee, B. L. (1988b) *Biochemistry* (in press).
Palmer, K. A., Scheraga, H. A., Riordan, J. F., & Vallee, B. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1965-1969.
Raetz, C. R. H., & Auld, D. S. (1972) *Biochemistry 11*, 2229-2236.
Richards, F. M., & Wyckoff, H. W. (1971) *Enzymes (3rd Ed.) 4*, 647-806.
Richards, F. M., & Wyckoff, H. W. (1973) *Atlas of Molecular Structures in Biology* (Phillips, D. C., & Richards, F. M., Eds.) Vol. 1, Oxford University Press, London.
Roberts, G. C. K., Dennis, E. A., Meadows, D. H., Cohen, J. S., & Jardetzky, O. (1969) *Proc. Natl. Acad. Sci. U.S.A. 62*, 1151-1158.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Sela, M., & Anfinsen, C. B. (1957) *Biochim. Biophys. Acta 24*, 229-235.
Shapiro, R., & Vallee, B. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2238-2241.
Shapiro, R., Riordan, J. F., & Vallee, B. L. (1986) *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. (1988a) *Anal. Biochem.* (in press).
Shapiro, R., Strydom, D. J., Weremowicz, S., & Vallee, B. L. (1988b) *Biochem. Biophys. Res. Commun. 156*, 530-536.
St. Clair, D. K., Rybak, S. M., Riordan, J. F., & Vallee, B. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8330-8334.
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.
Tabor, S., & Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4767-4771.
Wlodawer, A., Miller, M., & Sjolin, L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3628-3631.
Wlodawer, A., Svensson, L. A., Sjolin, L., & Gilliland, G. L. (1988) *Biochemistry 27*, 2705-2717.

Purification and Immunochemical Characterization of the Cytoplasmic Androgen-Binding Protein of Rat Liver†

William F. Demyan, Fazlul H. Sarkar,‡ C. V. Ramana Murty,§ and Arun K. Roy*
Division of Molecular Genetics, Department of Obstetrics and Gynecology, and Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284
Received June 29, 1988; Revised Manuscript Received October 28, 1988

ABSTRACT: The cytoplasmic androgen-binding (CAB) protein of the male rat liver has been implicated to play a role in the androgen-dependent regulation of α_{2u}-globulin synthesis. The liver of the adult male rat contains about 50 fmol of specific high-affinity androgen-binding activity per milligram of total cytosolic protein. Photoaffinity labeling with [^{3}H]R-1881 followed by SDS–polyacrylamide gel electrophoresis and autoradiography shows that the CAB is a 31-kilodalton protein. By means of DEAE-cellulose chromatography and preparative SDS–polyacrylamide gel electrophoresis, we have purified the CAB protein to electrophoretic homogeneity and have raised polyclonal rabbit antiserum that is monospecific to this protein. In the sucrose density gradient, the antiserum reacted with the androgen-binding component of the male liver cytosol prelabeled with tritiated dihydrotestosterone. Western blot analysis of the liver cytosol showed that the antiserum recognizes only the 31-kDa androgen-binding component. Such immunoblotting also showed that unlike the young adult, the androgen-insensitive states during prepuberty and senescence are associated with a marked reduction in the hepatic concentration of the immunoreactive CAB protein. No immunochemical cross-reactivity between CAB and another androgen-binding component of M_r 29K (which is associated with androgen insensitivity during prepuberty and senescence) was observed. The latter finding favors the possibility that 31- and 29-kDa androgen-binding components may have distinct sequence structure.

A number of hepatic genes are differentially expressed in the male and female liver (Roy & Chatterjee, 1983; Gustafsson et al., 1983). In addition, synthesis of these sex-dependent proteins undergoes changes during maturation and aging (Roy et al., 1983a; Richardson et al., 1987). In the rat, the male-specific urinary protein α_{2u}-globulin is one of the major sexually dimorphic secretory proteins of hepatic origin (Roy et al., 1983a; Dolan et al., 1983). α_{2u}-Globulin is normally absent in the liver of female rats, and, in the case of males, its hepatic synthesis begins at puberty (~40 days) and reaches a peak level at about 75–85 days; the protein level

† This work was supported by NIH Grant DK-14744.
* Address correspondence to this author at the Division of Molecular Genetics, Department of Obstetrics and Gynecology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78284.
‡ Present address: Henry Ford Hospital, Detroit, MI 48202.
§ Present address: Wyeth-Ayerst Research, P.O. Box 8299, Philadelphia, PA 19101.

0006-2960/89/0428-1732$01.50/0