Puca, G. A., Abbondanza, C., Nigro, V., Armetta, I., Medici, N., & Molinari, A. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5367–5371.
Sabbah, M., Redeuilh, G., Secco, C., & Baulieu, E.-E. (1987) *J. Biol. Chem. 262*, 8631–8635.
Sakaue, Y., & Thompson, E. B. (1977) *Biochem. Biophys. Res. Commun. 77*, 533–541.
Schmidt, T. J., & Littwack, G. (1982) *Physiol. Rev. 62*, 1131–1192.
Schmidt, T. J., Harmon, J. M., & Thompson, E. B. (1980) *Nature (London) 286*, 507–510.
Shyamala, G. (1975) *Biochem. Biophys. Res. Commun. 64*, 408–415.
Weinberger, C., Thompson, C. C., Ong, E. S., Lebo, R., Gruol, D. J., & Evans, R. M. (1986) *Nature (London) 324*, 641–646.
Young, M., & Koroly, M. J. (1980) *Biochemistry 19*, 5316–5321.

Tryptophan Fluorescence as a Probe of Placental Ribonuclease Inhibitor Binding to Angiogenin†

Frank S. Lee, David S. Auld, and Bert L. Vallee*

Center for Biochemical and Biophysical Sciences and Medicine, Department of Pathology, and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115

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ABSTRACT: The binding of human placental ribonuclease inhibitor (PRI) to angiogenin, a human protein that induces neovascularization, occurs with a 1:1 stoichiometry and is accompanied by a 50% increase in tryptophan fluorescence. In contrast, the binding of PRI to bovine pancreatic RNase A or to angiogenin oxidized at its single tryptophan residue results in a quenching of fluorescence. These observations suggest that there is a change in the local environment of Trp-89 of angiogenin. Quenching experiments with acrylamide are consistent with the view that Trp-89 is exposed in the native protein and becomes less accessible upon formation of the complex with PRI. Stopped-flow kinetic measurements monitoring the fluorescence enhancement indicate a two-step mechanism for the binding of PRI to angiogenin. The first step involves rapid formation of an enzyme·inhibitor complex, EI, followed by a slower isomerization of EI to a tight enzyme·inhibitor complex, EI*:

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

In 0.1 M NaCl at pH 6 and 25 °C, the values of K_1 and k_2 are 0.53 μM and 97 s^{-1}, respectively. The apparent second-order rate constant of association at protein concentrations $\ll K_1$ is approximated by k_2/K_1 and equals 1.8×10^8 M^{-1} s^{-1}. The corresponding value for the association of PRI with RNase A is only slightly higher, 3.4×10^8 M^{-1} s^{-1}. The effects of pH and sodium chloride concentration on the association rate of PRI with angiogenin suggest the importance of ionizable groups and ionic interactions, respectively, in the association process. Increasing the pH from 5.5 to 9 decreases the apparent second-order rate constant of association of PRI with angiogenin 13-fold; increasing the sodium chloride concentration from 75 mM to 1 M decreases it 140-fold.

Several angiogenic substances have been isolated recently (Vallee et al., 1985; Folkman & Klagsbrun, 1987). Little is currently known, however, about the regulation of these substances. Angiogenin is a potent blood vessel inducing protein that has been obtained from both the conditioned medium of the human adenocarcinoma cell line HT-29 and normal human plasma (Fett et al., 1985; Shapiro et al., 1987). It is remarkably homologous to RNase A[1] and, indeed, exhibits ribonucleolytic activity (Strydom et al., 1985; Shapiro et al., 1986a; St. Clair et al., 1987). An RNase inhibitor isolated from human placenta (PRI) (Blackburn et al., 1977) inhibits both the angiogenic and ribonucleolytic activities (K_i < 0.1 nM) of angiogenin (Shapiro & Vallee, 1987). Thus, PRI may play a critical role in their physiological control.

PRI is a member of a family of RNase inhibitors that occur in the tissues of many mammalian species (Roth, 1967; Blackburn & Moore, 1982). Other members of this family have been purified to homogeneity from bovine brain and liver and porcine, ovine, and rodent livers (Burton et al., 1980;

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* Address correspondence to this author at the Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, Boston, MA 02115.

[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; PRI, placental ribonuclease inhibitor; HSA, human serum albumin; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; C>p, cytidine cyclic 2′,3′-phosphate; CpG, cytidylyl(3′–5′)guanosine; UpG, uridylyl(3′–5′)guanosine; CD, circular dichroism, HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; DMS, dimethyl sulfide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; *n*, number of determinations.

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Burton & Fucci, 1982). These inhibitors are acidic cytoplasmic proteins of $M_r \sim 50000$ that form 1:1 complexes with both intracellular and secretory ribonucleases. It has been speculated that these inhibitors may play a role in controlling cytoplasmic RNA levels (Imrie & Hutchison, 1965; Kraft & Shortman, 1970).

The interaction of PRI with angiogenin is important for an understanding of the in vivo role of PRI in the regulation of angiogenin and an exploration of novel approaches to antiangiogenesis. The conservation of Tyr-92 in RNases of different species, which all bind PRI, and the results of CD measurements of RNase A derivatives led to the postulate that Tyr-92 may play a key role in the RNase A·PRI interaction (Blackburn & Gavilanes, 1980). In angiogenin, the sole tryptophan residue in the molecule, Trp-89, substitutes for this tyrosine (Strydom et al., 1985). These observations prompted us to examine tryptophan fluorescence as a possible probe of the interaction between angiogenin and PRI. The present paper describes tryptophan fluorescence enhancement in the angiogenin·PRI complex, evidence that enhancement of angiogenin Trp-89 fluorescence contributes to this, and the results of experiments using this fluorescence change to study the kinetic mechanism of PRI binding to angiogenin.

Experimental Procedures

Materials. C>p, CpG, UpG, Tris, EDTA, and human serum albumin (HSA) were obtained from Sigma Chemical Co., DTT and Hepes were from Research Organics, DMSO was from Fisher, DMS and indole were from Aldrich Chemical Co., and acrylamide was from Bio-Rad. Mes was obtained from Calbiochem and did not inhibit RNase A at 0.1 M, the concentration employed in RNase A assays. Mes from several other sources markedly inhibited RNase A activity. All buffers were degassed before use.

Proteins. Angiogenin was obtained from human plasma (Shapiro et al., 1987). Its concentration was determined by amino acid analysis (Picotag, Waters Associates). Bovine pancreatic RNase A (code RAF) was purchased from Cooper Biomedical. RNase A concentration was determined spectrophotometrically with a molar absorptivity of 9800 M^{-1} cm^{-1} at 278 nm (Sela & Anfinsen, 1957). PRI was isolated as described (Blackburn, 1979). Its concentration was determined by inhibition of RNase A activity toward C>p (Blackburn, 1979) with a titration plot consisting of three to four points. These plots are linear under the conditions employed; i.e., $[PRI] \gg K_i$ for the inhibition of RNase A.

Fluorescence Measurements. Fluorescence data were recorded at 25 °C on a Perkin-Elmer Model MPF-3 fluorescence spectrophotometer equipped with a Model 150 xenon lamp and a Hitachi Model QPD33 recorder. A 3-mL cuvette of 1-cm path length was used for recording fluorescence spectra. Excitation was at 285 nm, and emission was monitored with a 310-nm cutoff filter.

For fluorescence quenching measurements, small aliquots of a stock acrylamide solution were added to protein solutions in a cylindrical cuvette of 0.5-cm path length. Excitation was at 295 nm, and emission was monitored at the emission λ_{max} with a 310-nm cutoff filter. The absorbance was corrected for the added acrylamide (Lakowicz, 1983) by using a value of 0.25 M^{-1} cm^{-1} for the molar absorptivity of acrylamide at 295 nm (Eftink & Ghiron, 1981). Quenching of fluorescence was analyzed by the Stern–Volmer equation:

$$F_o/F = 1 + K_{sv}[Q]$$

where F_o and F are the fluorescence intensities in the absence and presence of quencher, respectively, [Q] is the concentration of quencher, and K_{sv} is the Stern–Volmer quenching constant. K_{sv} was obtained from the initial slope of the Stern–Volmer plot.

Tryptophan Modification. The tryptophan of angiogenin was oxidized by a modification of the method of Savige and Fontana (1977). Angiogenin was treated with 0.4 M DMSO and 8 M HCl for 1 h at 25 °C followed by 0.4 M DMS and 8 M HCl for 1 h at 25 °C. The first reaction oxidizes tryptophan and methionine to oxindolylalanine and methione sulfoxide, respectively; the second reverses the methionine oxidation (Shechter, 1986). Each reaction was quenched by gel filtration chromatography in 50 mM acetic acid on Sephadex G-25, 4 °C. In order to remove protein that had been cleaved internally by the modification, the final product was applied to a Synchropak RP-P C18 reversed-phase HPLC column (250 × 4.1 mm; Synchrom, Inc.) previously equilibrated with a 7:3 mixture of 0.1% TFA (solvent A) and 2-propanol/acetonitrile/water (3:2:2 v/v/v) containing 0.08% TFA (solvent B). A Waters Associates liquid chromatography system equipped with a 214-nm detector and a Hewlett-Packard 3390A integrator was employed. Elution was performed with a 54-min linear gradient from 30% to 42% solvent B at a flow rate of 1 mL/min. A peak containing material that was more than 95% uncleaved, as judged by SDS–PAGE under reducing conditions (Laemmli, 1970), was subjected to further analysis. Tryptophan analysis was performed as described (Shapiro et al., 1986b).

Stopped-Flow Measurements. Stopped-flow kinetic measurements were made at 25 °C on a Durrum-Gibson Model D-110 stopped-flow spectrophotometer equipped with a 75-W xenon–Hg lamp and an EM-9526QB (S13) photomultiplier. Excitation was at 285 nm, and emission was monitored with a band-pass filter centered at 340 nm. Fluorescence changes were stored digitally, 1000 data points per experiment, on floppy disks by a DEC PDP 11/34 computer equipped with an AR11 A/D converter, a VT-55 Decscope, and a Decwriter III printer.

In a typical experiment, angiogenin and PRI were diluted separately into 0.1 M Mes, pH 6, containing 0.1 M NaCl and 1 mM EDTA immediately before filling the syringe chambers. Equal volumes (200 μL) of buffered angiogenin and PRI were mixed at 25 °C for each run. Either the angiogenin or PRI was in 4–12-fold molar excess over the other component, thus giving pseudo-first-order rate conditions. The first one to three half-lives of the reaction, represented by 100–300 data points, were used to obtain the rate constant. In all cases, data analysis was performed over a time period during which there was less than a 15% decrease in the component present in molar excess. Pseudo-first-order rate constants were determined by linear regression of $-\log (F_\infty - F_t)$ versus time, where F is fluorescence intensity. These plots were linear in all cases. Each value of the pseudo-first-order rate constant k_{obsd} represents an average of at least three determinations.

RNase A Assays. The apparent second-order rate constant for the association of PRI with RNase A was determined by an experiment examining the competition between RNase A and angiogenin for PRI. PRI was added to a mixture of 1 equiv of RNase A and 0.67–4.8 equiv of angiogenin at 25 °C. Buffer was 0.1 M Mes, pH 6, containing 0.1 M NaCl and 1 mM EDTA. The solution was mixed, and after 15 s, 12 μL of substrate was added to give a final concentration of 100 μM in 600 μL. Substrate was either CpG or UpG (Witzel & Barnard, 1962) when the final total PRI concentration was either 3 or 15 nM, respectively. The activity of free RNase A was then determined by following the decrease in absorbance

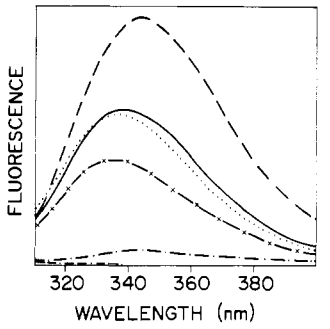

FIGURE 1: Fluorescence emission spectra of angiogenin (·–·), RNase A (·––·), PRI (—), angiogenin·PRI (– – –), RNase A·PRI (···), and DMSO/HCl-oxidized angiogenin·PRI (–×–). Excitation was at 285 nm. All proteins were 0.1 μM in 0.1 M Mes, pH 6, containing 0.1 M NaCl and 1 mM EDTA at 25 °C.

at 286 (CpG) or 280 nm (UpG) on a Varian Model 219 spectrophotometer. In control assays, both angiogenin and PRI were omitted.

Results

Fluorescence Spectra. The fluorescence spectra of angiogenin, RNase A, PRI, and several PRI complexes are shown in Figure 1. PRI contains six tryptophans (F. Lee and E. Fox, unpublished experiments) and has a fluorescence spectrum with a maximum at 338 nm. That of angiogenin, with one tryptophan, is much weaker, while that of RNase A, without tryptophans but with six tyrosines, is negligible at 340 nm. The fluorescence of the angiogenin·PRI complex is enhanced 50% relative to that of the sum of the two proteins; the maximum at 343 nm is virtually identical with that of angiogenin alone. The enhancement observed upon addition of angiogenin to PRI indicates a 1:1 stoichiometry (data not shown). The fluorescence of the RNase A·PRI complex is quenched 5%.

Tryptophan Modification. The Trp-89 of angiogenin was oxidized by means of DMSO and HCl to examine the contribution of this residue to the fluorescence of the angiogenin·PRI complex. This abolished angiogenin tryptophan fluorescence to less than 5%, and amino acid analysis indicated oxidation of the one tryptophan without changes in any other residues. Compared with the fluorescence of PRI alone, that of the complex between this modified angiogenin and PRI is quenched 33% (Figure 1). Kinetic data show that the modified angiogenin derivative binds more weakly to PRI than does native angiogenin but should form the 1:1 complex under the conditions employed (F. Lee, unpublished experiments).

Quenching of Tryptophan Fluorescence by Acrylamide. Figure 2 shows Stern–Volmer plots for tryptophan fluorescence quenching in angiogenin, PRI, and several PRI complexes. The fluorescence of the single tryptophan of angiogenin is quenched with a K_{sv} value of 20.1 M^{-1} and that of indole with a K_{sv} value of 40.9 M^{-1}. The fluorescence of PRI, the angiogenin·PRI complex, the RNase A·PRI complex, and the DMSO/HCl-oxidized angiogenin·PRI complex are all quenched with significantly lower K_{SV} values. Thus, those for PRI alone and the angiogenin·PRI complex are identical and equal 7.2 M^{-1}; that for the RNase A·PRI complex is 5.0 M^{-1}. Acrylamide, 0.4 M, affected neither the activity of RNase A toward C>p nor the capacity of 1 equiv of PRI to completely inhibit RNase A in this assay.

Stopped-Flow Kinetics of Interaction of PRI with Angiogenin. The fluorescence enhancement accompanying the binding of PRI to angiogenin was monitored as a means of studying the pre-steady-state kinetics of this interaction. Pseudo-first-order kinetics are observed for either excess angiogenin or excess PRI.

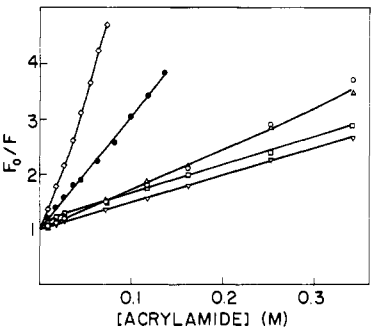

FIGURE 2: Stern–Volmer plots for quenching by acrylamide of fluorescence of indole (◊) and of tryptophan in angiogenin (●), PRI (○), angiogenin·PRI (△), RNase A·PRI (▽), and DMSO/HCl-oxidized angiogenin·PRI (□). Excitation was at 295 nm. Emission was monitored at λ_{max} of emission. Proteins were 0.15–4 μM in 10 mM Tris, pH 7.5, 25 °C.

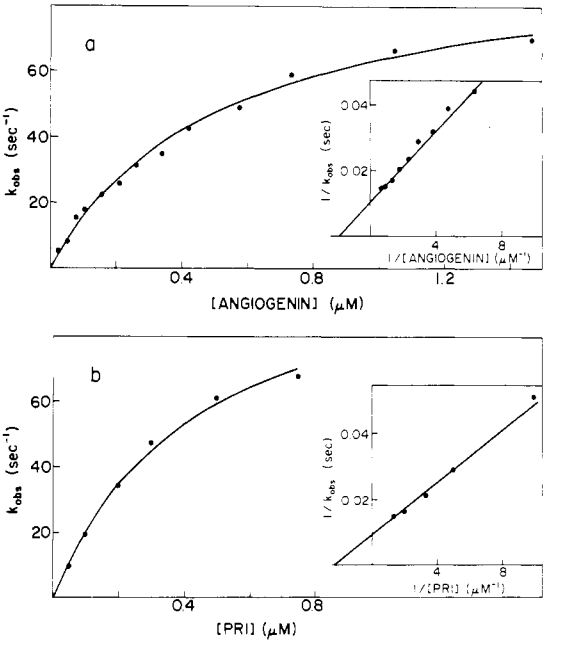

FIGURE 3: Dependence of the pseudo-first-order rate constant, k_{obsd}, on (a) [angiogenin] and (b) [PRI]. Tryptophan fluorescence was monitored with a 340-nm band-pass filter. Excitation was at 285 nm. Buffer was 0.1 M Mes, pH 6, containing 0.1 M NaCl and 1 mM EDTA at 25 °C. The curved lines are calculated from eq 1 and the parameters (a) K_1 = 0.53 μM and k_2 = 97 s^{-1} and (b) K_1 = 0.45 μM and k_2 = 112 s^{-1} obtained by analysis of the data at [angiogenin] or [PRI] ≥ 0.1 μM (insets).

The dependence of the pseudo-first-order rate constant, k_{obsd}, on angiogenin concentration and PRI concentration in 0.1 M NaCl at pH 6 is shown in Figure 3, parts a and b. The following mechanism is consistent with the observed concentration dependence:

$$E + I \underset{k_{-1}\ (fast)}{\overset{k_1\ (fast)}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^*$$

where E is angiogenin, I is PRI, and EI* refers to a different form of the EI complex. The predicted pseudo-first-order rate constant for this mechanism is

$$k_{obsd} = k_{-2} + \frac{k_2[E]}{K_1 + [E]} \qquad [E] \gg [I] \qquad (1)$$

where $K_1 = k_{-1}/k_1$. This equation also applies when [I] ≫ [E], with the substitution of [I] for [E].

Since k_{-2} for the angiogenin·PRI complex is extremely low (Lee et al., 1989), it can be neglected for these calculations. A double-reciprocal plot of $1/k_{obsd}$ versus either 1/[angiogenin] or 1/[PRI] linearizes the data (Figure 3, parts a and b, insets).

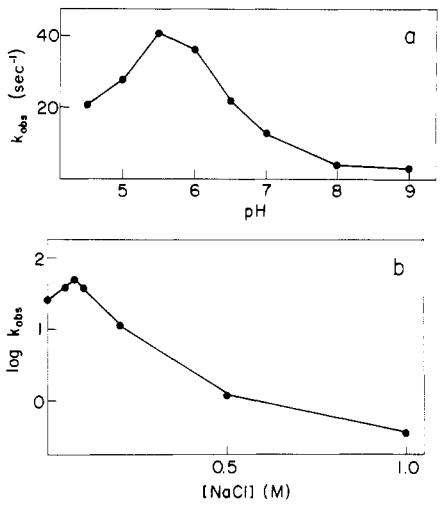

FIGURE 4: Effect of (a) pH and (b) sodium chloride concentration on k_{obsd}. Angiogenin and PRI concentrations were 0.100 and 0.025 μM, respectively. (a) Buffers were 10 mM acetate (pH 4.5 and 5), Mes (pH 5.5–6.5), Hepes (pH 7), and Tris (pH 8 and 9), containing 0.1 M NaCl and 1 mM EDTA at 25 °C. (b) Buffer was 10 mM Mes, pH 6, containing 1 mM EDTA at 25 °C.

Variation of angiogenin concentration yields values for K_1 and k_2 of 0.53 μM and 97 s^{-1}, respectively. At concentrations of angiogenin and PRI $\ll K_1$, the apparent second-order rate constant of association is approximated by k_2/K_1 and equals 1.8×10^8 M^{-1} s^{-1}. From the variation of PRI concentration, the values of K_1 and k_2 are 0.45 μM and 112 s^{-1}, respectively.

Figure 4 shows the variation of the pseudo-first-order rate constant, k_{obsd}, with pH and [NaCl]. The concentrations of both angiogenin and PRI were at least 5-fold lower than the K_1 value for the first step in the mechanism, so that $k_{obsd} \simeq (k_2/K_1)$[E]. Thus, changes in k_{obsd} could reflect changes in k_2, K_1, or both. The pH profile of k_{obsd} displays a maximum at pH 5.5 (Figure 4a); the calculated value of k_2/K_1 is 4.1×10^8 M^{-1} s^{-1}. At pH 9, the corresponding value is 13-fold less. The NaCl concentration at which k_{obsd} is maximal at pH 6 is 75 mM (Figure 4b); the value of k_2/K_1 is 5.0×10^8 M^{-1} s^{-1}. In 1 M NaCl, the corresponding value is 140-fold less.

Rate of Association of RNase A with PRI. The rate of association of RNase A with PRI was determined as a basis for comparison with angiogenin as well as a means of obtaining the K_i value for the inhibition of RNase A (Lee et al., 1989). Since the change in fluorescence upon formation of the RNase A·PRI complex (Figure 1) is not large enough to allow its use for determination of the rate of association, this rate was determined by examining the competition between RNase A and angiogenin for PRI.

PRI was added to a mixture of RNase A and angiogenin, and the degree of partitioning of PRI between RNase A and angiogenin was determined by assay of free RNase A. Under the conditions of the assay, angiogenin does not cleave CpG or UpG, the substrates employed, at a detectable rate (Shapiro et al., 1986a). In addition, k_{-2} can be neglected (Lee et al., 1989); hence, the partitioning of PRI between RNase A and angiogenin only reflects the association rates. If the association of PRI with RNase A also proceeds by the two-step mechanism described for angiogenin, then the following scheme applies:

$$\begin{array}{ccccc} & & & & \\ R & \underset{K_{1,R}}{\rightleftharpoons} & R\cdot PRI & \xrightarrow{k_{2,R}} & R\cdot PRI^* \\ & PRI & & & \\ A & \rightleftharpoons & A\cdot PRI & \xrightarrow{k_{2,A}} & A\cdot PRI^* \\ & K_{1,A} & & & \end{array}$$

where R is RNase A and A is angiogenin. The rate equation for the formation of the R·PRI* complex is

$$\frac{d[R\cdot PRI^*]}{dt} = k_{2,R}[R\cdot PRI] = \frac{k_{2,R}([R]_T - [R\cdot PRI^*])[PRI]}{K_{1,R} + [PRI]}$$

where $[R]_T$ is the total RNase A concentration. An analogous equation applies for the association of angiogenin with PRI. In this experiment, the highest PRI concentration used is 15 nM, 35-fold less than $K_{1,A}$. If [PRI] is also $\ll K_{1,R}$, then

$$\frac{d[A\cdot PRI^*]}{d[R\cdot PRI^*]} = \frac{(k_{2,A}/K_{1,A})([A]_T - [A\cdot PRI^*])}{(k_{2,R}/K_{1,R})([R]_T - [R\cdot PRI^*])}$$

$$\alpha = \frac{\ln([A]_T/[A]_F)}{\ln([R]_T/[R]_F)} = \frac{k_{2,A}/K_{1,A}}{k_{2,R}/K_{1,R}}$$

where $[A]_F$ and $[R]_F$ are free angiogenin and RNase A concentrations, respectively. The same equation applies if the association of PRI with RNase A is a one-step mechanism, with the substitution of the second-order rate constant of association of PRI with RNase A for $k_{2,R}/K_{1,R}$ (Vincent & Lazdunski, 1972).

Experimentally, free RNase A concentration is determined by enzymatic activity. Free angiogenin concentration is then calculated by assuming that any PRI not bound to RNase A is bound to angiogenin. The experimental value of α, 0.53, combined with the apparent second-order rate constant of association of 1.8×10^8 M^{-1} s^{-1} for angiogenin and PRI gives an apparent second-order rate constant of 3.4×10^8 M^{-1} s^{-1} for RNase A and PRI. The value of α when [PRI] = 15 nM, 0.53 ± 0.043 ($n = 8$), is indistinguishable from that when [PRI] is 5-fold lower, 0.52 ± 0.073 ($n = 6$), supporting the assumption that [PRI] $\ll K_{1,R}$.

Discussion

The inhibition of both the enzymatic and angiogenic activities of angiogenin by PRI suggests a potentially critical function of PRI in the control of these activities (Shapiro & Vallee, 1987). While the RNase A·PRI interaction has been explored through the binding of PRI to proteolytically (Blackburn & Jailkhani, 1979) and chemically (Blackburn & Jailkhani, 1979; Blackburn & Gavilanes, 1980) modified RNase A derivatives, nothing is currently known about either the structural basis or kinetic mechanism of the angiogenin·PRI interaction. We have therefore examined tryptophan fluorescence both as a probe of changes in tryptophan environments in the angiogenin·PRI complex and as a means of monitoring the pre-steady-state kinetics of the angiogenin·PRI interaction.

The tryptophan fluorescence of the angiogenin·PRI complex is significantly enhanced (Figure 1) while that of the RNase A·PRI complex is quenched slightly. Since angiogenin contains one tryptophan whereas RNase A has none, the enhancement of fluorescence in the angiogenin·PRI complex suggests that enhancement of angiogenin Trp-89 fluorescence has occurred in the angiogenin·PRI complex. This, in turn, suggests that the environment of angiogenin Trp-89 has changed markedly in the complex.

Two additional lines of evidence support this view. The first is the spectral consequence of chemical modification of Trp-89. The fluorescence of the complex between PRI and angiogenin oxidized at Trp-89 is quenched (Figure 1). The molecular mechanisms underlying changes in fluorescence of tryptophan residues are undoubtedly complex and include changes in their local environments and specific interactions with other residues (Longworth, 1983). Nevertheless, the opposite effects on

tryptophan fluorescence observed with native and oxidized angiogenin suggest that enhancement of fluorescence of angiogenin Trp-89 has occurred in the complex.

The second is the result of tryptophan fluorescence quenching experiments using acrylamide (Figure 2). Fluorescence quenching by acrylamide has been used to assess the degree of exposure of tryptophan residues in proteins (Eftink & Ghiron, 1976): the higher the Stern–Volmer quenching constant, K_{sv}, the greater the tryptophan exposure. The high K_{sv} value for angiogenin Trp-89, 20.1 M^{-1}, suggests that it is exposed. A precise K_{sv} value for angiogenin Trp-89 in the angiogenin·PRI complex cannot be ascertained, since the contribution of PRI tryptophan quenching to that of the complex cannot be determined exactly. Nevertheless, the following suggests that in the complex this K_{sv} value is lower, indicative of a decreased accessibility to acrylamide: (a) the Stern–Volmer plots for PRI alone and its complex with the DMSO/HCl-oxidized angiogenin are similar (Figure 2) and suggest that acrylamide quenches the tryptophans of PRI to a similar extent when PRI is either free or complexed with angiogenin (b) the K_{sv} value for the angiogenin·PRI complex, 7.2 M^{-1}, is significantly lower than that for free angiogenin.

Two ways by which the environment of Trp-89 in angiogenin might change upon formation of the complex are by contact of this residue with PRI or by its burial in the angiogenin molecule itself. Indirect evidence for the former comes from previous studies on the RNase A·PRI interaction (Blackburn & Gavilanes, 1982). PRI protects Lys-91 in RNase A from modification by methyl acetimidate. This lysine is adjacent to Tyr-92, the residue for which Trp-89 substitutes in angiogenin (Strydom et al., 1985). Therefore, if the overall tertiary structures of angiogenin and RNase A are similar, as suggested by energy minimization calculations (Palmer et al., 1986), and the contact regions between PRI and the two molecules are conserved [see Shapiro and Vallee (1987)], then Trp-89 would be expected to be close to, or in, the contact region with PRI. Formation of the complex could therefore lead to a marked change in Trp-89 fluorescence.

The observed dependence of k_{obsd} on angiogenin or PRI concentration (Figure 3) is consistent with a two-step mechanism that involves a rapid equilibrium resulting in formation of an EI complex followed by a slower isomerization of EI to form a tight EI* complex;[2] the data eliminate the obvious alternative mechanisms, such as a one-step binding or a slow isomerization in either enzyme or inhibitor followed by rapid association with either inhibitor or enzyme, respectively [see mechanisms A and C in Shapiro and Riordan (1984)]. The mechanism consistent with the data has been postulated to occur in the interaction of other enzymes with tight-binding inhibitors (Morrison & Walsh, 1988). Examples include the inhibition of trypsin by soybean trypsin inhibitor (Luthy et al., 1973), α-chymotrypsin by pancreatic trypsin inhibitor (Quast et al., 1974), dihydrofolate reductase by methotrexate (Williams et al., 1979), and angiotensin-converting enzyme by captopril and enalapril (Shapiro & Riordan, 1974; Bull et al., 1985). With the serine proteases, small conformational changes in the inhibitor have been observed in the X-ray crystal structures of several enzyme·inhibitor complexes; thus, the second step may involve a small conformational change in these inhibitors (Huber & Bode, 1978; Laskowski & Kato, 1980).

The apparent second-order rate constant, k_2/K_1, for the association of angiogenin with PRI at protein concentrations $\ll K_1$ is 1.8×10^8 M^{-1} s^{-1}. This value is close to the diffusion-controlled limit and is high in comparison to values reported for other protein–protein interactions. The association of serine proteases with proteinase inhibitors, e.g., occurs with rate constants in the general range of 10^5–10^7 M^{-1} s^{-1} (Travis & Salvesen, 1983).

The pH dependence of the pseudo-first-order rate constant, k_{obsd}, suggests that ionizable groups on either angiogenin or PRI, or both, are involved in the association between the two proteins (Figure 4a). Similarly, the sodium chloride concentration dependence of k_{obsd} suggests that ionic interactions play a significant role in the association between angiogenin and PRI (Figure 4b). The binding involves the interaction of angiogenin, a very basic protein (pI > 9.5; Fett et al., 1985), with PRI, an acidic one (pI = 4.7; Blackburn et al., 1977). Thus, ionic interactions might be expected to play an important role in the binding of PRI to angiogenin.

The apparent second-order rate constant of association of RNase A and PRI, 3.4×10^8 M^{-1} s^{-1}, is slightly higher than that for angiogenin and PRI and is similarly close to the diffusion-controlled limit. This finding contrasts with a previous report that porcine thyroid ribonuclease inhibitor is a slow binding inhibitor of RNase A (Turner et al., 1983). The source of this inhibitor is different, and the substrate employed, yeast tRNA, may also complicate comparison of association rates.

The association kinetics of angiogenin with PRI may have implications for the physiological inhibition of angiogenin. The rate of association of PRI with angiogenin relative to that of PRI with other RNases will determine the partitioning of PRI between angiogenin and competing RNases, if PRI is present initially in uncomplexed form and the lifetimes of the complexes are long relative to those in vivo (determined by the in vivo clearance rates). It follows that the ratio of free to bound angiogenin may be influenced by the presence of other PRI-binding RNases. It also suggests that the dissociation rate of the angiogenin·PRI complex may play as important a role as the association rate. This parameter is also critical for the determination of the K_i value of the angiogenin inhibition by PRI and will be examined in greater detail in the following paper (Lee et al., 1989).

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Registry No. PRI, 39369-21-6; Trp, 73-22-3; RNase, 9001-99-4.

References

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

Blackburn, P., & Jailkhani, B. L. (1979) *J. Biol. Chem. 254*, 12488–12493.

Blackburn, P., & Gavilanes, J. G. (1980) *J. Biol. Chem. 255*, 10959–10965.

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.

[2] The following kinetically equivalent mechanism is also consistent with the observed concentration dependence:

$$EI \underset{k_{-1}\,(fast)}{\overset{k_1\,(fast)}{\rightleftharpoons}} E + I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^*$$

The apparent second-order rate constant of association of PRI with angiogenin and K_i value for PRI binding to angiogenin are the same for both mechanisms.

Bull, H. G., Thornberry, N. A., Cordes, M. H. J., Patchett, A. A., & Cordes, E. H. (1985) *J. Biol. Chem. 260*, 2952–2962.

Burton, L. E., & Fucci, N. P. (1982) *Int. J. Pept. Protein Res. 19*, 372–379.

Burton, L. E., Blackburn, P., & Moore, S. (1980) *Int. J. Pept. Protein Res. 16*, 359–364.

Eftink, M. R., & Ghiron, C. A. (1976) *Biochemistry 15*, 672–680.

Eftink, M. R., & Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199–227.

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.

Folkman, J., & Klagsbrun, M. (1987) *Science (Washington, D.C.) 235*, 442–447.

Huber, R., & Bode, W. (1978) in *Proceedings of the 11th FEBS Meeting on Regulatory Proteolytic Enzymes and Their Inhibitors* (Magnusson, S., Ottesen, M., Foltmann, B., Dano, K., & Neurath, H., Eds.) pp 15–34, Pergamon, Oxford.

Imrie, R. C., & Hutchison, W. C. (1965) *Biochim. Biophys. Acta 108*, 106–113.

Kraft, N., & Shortman, K. (1970) *Biochim. Biophys. Acta 217*, 164–175.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, pp 43–47, Plenum, New York.

Laskowski, M., Jr., & Kato, I. (1980) *Annu. Rev. Biochem. 49*, 593–626.

Lee, F. S., Shapiro, R., & Vallee, B. L. (1989) *Biochemistry* (following paper in this issue).

Longworth, J. W. (1983) in *Time-Resolved Fluorescence Spectroscopy in Biochemistry and Biology* (Cundall, R. B., & Dale, R. E., Eds.) pp 651–686, Plenum, New York.

Luthy, J. A., Praissman, M., Finkenstadt, W. R., & Laskowski, M., Jr. (1973) *J. Biol. Chem. 248*, 1760–1771.

Morrison, J. F., & Walsh, C. T. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol. 61*, 201–301.

Palmer, K. A., Scheraga, H. A., Riordan, J. F., & Vallee, B. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1965–1969.

Quast, U., Engel, J., Heumann, H., Krause, G., & Steffen, E. (1974) *Biochemistry 13*, 2512–2520.

Roth, J. S. (1967) *Methods Cancer Res. 3*, 153–242.

Savige, W. E., & Fontana, A. (1977) *Methods Enzymol. 47*, 442–453.

Sela, M., & Anfinsen, C. B. (1957) *Biochim. Biophys. Acta 24*, 229–235.

Shapiro, R., & Riordan, J. F. (1984) *Biochemistry 23*, 5234–5240.

Shapiro, R., & Vallee, B. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2238–2241.

Shapiro, R., Riordan, J. F., & Vallee, B. L. (1986a) *Biochemistry 25*, 3527–3532.

Shapiro, R., Fett, J. W., Strydom, D. J., & Vallee, B. L. (1986b) *Biochemistry 25*, 7255–7264.

Shapiro, R., Strydom, D. J., Olson, K. A., & Vallee, B. L. (1987) *Biochemistry 26*, 5141–5146.

Shechter, Y. (1986) *J. Biol. Chem. 261*, 66–70.

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.

Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655–709.

Turner, P. M., Lerea, K. M., & Kull, F. J. (1983) *Biochem. Biophys. Res. Commun. 114*, 1154–1160.

Vallee, B. L., Riordan, J. F., Lobb, R. R., Higachi, N., Fett, J. W., Crossley, G., Buhler, R., Budzik, G., Breddam, K., Bethune, J. L., & Alderman, E. M. (1985) *Experientia 41*, 1–15.

Vincent, J. P., & Lazdunski, M. (1972) *Biochemistry 11*, 2967–2977.

Williams, J. W., Morrison, J. F., & Duggleby, R. G. (1979) *Biochemistry 18*, 2567–2573.

Witzel, H., & Barnard, E. A. (1962) *Biochem. Biophys. Res. Commun. 7*, 295–299.