Tight-Binding Inhibition of Angiogenin and Ribonuclease A by Placental Ribonuclease Inhibitor†

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ABSTRACT: The dissociation rate constant of the angiogenin·placental ribonuclease inhibitor complex was determined by measuring the release of free angiogenin from the complex in the presence of scavenger for free placental ribonuclease inhibitor (PRI). In 0.1 M NaCl, pH 6, 25 °C, this value is 1.3×10^{-7} s^{-1} ($t_{1/2} \simeq 60$ days). The K_i value for the binding of PRI to angiogenin, calculated from the association and dissociation rate constants, is 7.1×10^{-16} M. The corresponding values for the interaction of RNase A with PRI, determined by similar means, are both considerably higher: the dissociation rate constant is 1.5×10^{-5} s^{-1} ($t_{1/2} = 13$ h), and the K_i value is 4.4×10^{-14} M. Thus, PRI binds about 60 times more tightly to angiogenin than to RNase A. The effect of increasing sodium chloride concentration on the binding of PRI to RNase A was explored by Henderson plots. The K_i value increases to 39 pM in 0.5 M NaCl and to 950 pM in 1 M NaCl, suggesting the importance of ionic interactions. The mode of inhibition of RNase A by PRI was determined by examining the effect of a competitive inhibitor of RNase A, cytidine 2′-phosphate, on the association rate of PRI with RNase A. Increasing concentrations of cytidine 2′-phosphate decrease the association rate in a manner consistent with a competitive mode of inhibition.

Both the angiogenic and ribonucleolytic activities of angiogenin are abolished upon formation of its complex with placental ribonuclease inhibitor (PRI)[1] (Shapiro & Vallee, 1987). Information regarding the kinetic parameters that characterize the angiogenin·PRI interaction is critical to the elucidation of the biological role of PRI and the rational design of antiangiogenesis agents based on PRI. In this regard the K_i value is of central importance.

An upper limit of 0.1 nM was previously established for the K_i value reflecting the binding of PRI to angiogenin (Shapiro & Vallee, 1987). This tight-binding inhibition, together with the limitations of assays available for the ribonucleolytic activity of angiogenin (Shapiro et al., 1986a), precludes the use of standard kinetic approaches for the determination of the K_i value. Therefore, in order to calculate the K_i value for the inhibition of angiogenin by PRI, it is necessary to examine both the association and dissociation rate constants. The association rate constants have been determined in the preceding paper (Lee et al., 1989). The dissociation rate constant is determined here by examining the dissociation of the angiogenin·PRI complex in the presence of scavenger for free PRI. The data show the K_i value for the inhibition of angiogenin by PRI to be 0.71 fM. The corresponding value for RNase A is ~60-fold higher, and the mode of inhibition of RNase A by PRI is competitive.

Experimental Procedures

Materials. CpA, 2′-CMP, and pHMB were obtained from Sigma Chemical Co. The concentration of 2′-CMP was determined spectrophotometrically with a molar absorptivity of 7.6×10^3 M^{-1} cm^{-1} at 260 nm (Beaven et al., 1955). HT-RNase was isolated from conditioned media of the human colon adenocarcinoma cell line HT-29 as described (Shapiro et al., 1986b), and its concentration was determined by amino acid analysis. The sources of all other materials, including proteins, have been described previously (Lee et al., 1989). All buffers were degassed before use.

Dissociation Kinetics. (A) RNase A·PRI. The dissociation rate constant of the RNase A·PRI complex was obtained by two methods, which differ primarily in the means used to quantitate free RNase A. In the first method, RNase A was preincubated with 1.5 equiv of PRI for 20 min at 25 °C followed by the addition of 100 equiv of angiogenin as the scavenger. The buffer was 0.1 M Mes, pH 6, containing 0.1 M NaCl, 1 mM EDTA, and 5 mM DTT. The final concentration of RNase A was 0.73 μM. Aliquots of 90 μL were assayed periodically for free RNase A by injection onto a Synchropak CM300 cation-exchange HPLC column (250 × 4.1 mm; Synchrom, Inc.). A Waters Associates liquid chromatography system equipped with a 214-nm detector and a Hewlett-Packard 3390A integrator was used. Elution was achieved with a 30-min linear gradient from 0 to 0.8 M NaCl in 20 mM sodium phosphate, pH 6, at a flow rate of 1 mL/min. Free RNase A eluted at 15 min, well separated from the RNase A·PRI complex (breakthrough) and free angiogenin (22 min). Free RNase A was quantitated by peak area.

In the second method, RNase A was preincubated with 1.5 equiv of PRI for 20 min at 25 °C followed by the addition

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[1] Abbreviations: PRI, placental ribonuclease inhibitor; RNase A, bovine pancreatic ribonuclease A; HT-RNase, human tumor derived ribonuclease; 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; pHMB, *p*-(hydroxymercuri)benzoate; 2′-CMP, cytidine 2′-phosphate; CpA, cytidylyl(3′–5′)adenosine; CpG, cytidylyl(3′–5′)guanosine; HPLC, high-performance liquid chromatography; CM, carboxymethyl.

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of 20 equiv of HT-RNase as the scavenger. The buffer was the same as that described in the HPLC method except for the inclusion of 0.01% HSA. The final concentration of RNase A was 36 nM. Aliquots of 50 μL were assayed periodically for RNase A activity toward 150 μM CpG at 27 °C in 600 μL of 0.1 M Tris–acetate, pH 6.5, containing 1 mM EDTA. The decrease in absorbance at 286 nm was monitored continuously with a Gilford Model 250 spectrophotometer. CpG is cleaved over 1000 times more rapidly by RNase A than by HT-RNase (Shapiro et al., 1986b), and hence, the activity reflects only that of RNase A.

(*B*) *Angiogenin·PRI.* Angiogenin was preincubated with 1.5 equiv of PRI for 20 min at 25 °C followed by the addition of 250 equiv of RNase A as the scavenger. The buffer was the same as that described in the HPLC method for RNase A·PRI except for the use of 120 μM DTT. The final concentration of angiogenin was 1.41 μM. Free angiogenin, in 100-μL aliquots, was assayed by cation-exchange HPLC as above but employing a 15-min linear gradient from 0.2 to 0.6 M NaCl in 20 mM sodium phosphate, pH 7. Free angiogenin eluted at 11 min, while the angiogenin·PRI complex and free RNase A both appeared in the breakthrough. There was less than 2% volume loss in all samples during the experiment.

Henderson Plots. Assays measuring the inhibition of RNase A by PRI by Henderson plots employed the dinucleotide CpA as substrate (Witzel & Barnard, 1962). In a typical experiment, RNase A was preincubated with PRI for 80 min at 25 °C in 0.1 M Mes, pH 6, containing 0.1 M NaCl, 1 mM EDTA, 2 mM DTT, and 0.01% HSA followed by addition of CpA to give a final volume of 600 μL. Preincubation times up to 8 h resulted in the same degree of inhibition. Substrate cleavage was monitored continuously with a Varian Model 219 spectrophotometer at either 286 or 292 nm, depending on the substrate concentration. Initial velocities were measured during the first 8% of the reaction. Velocities at pH 7 and pH 8 were corrected for a relatively small absorbance drift which was observed in the absence of added enzyme, presumably due to oxidation of DTT. At pH 9, this drift comprised over 50% of the uninhibited initial velocity, and hence, DTT was omitted from the assay buffer.

Mode of Inhibition. PRI was added to a mixture of 1 equiv of RNase A, 0.8–4 equiv of angiogenin, and 0–50 μM 2′-CMP in 600 μL of 0.1 M Mes, pH 6, containing 0.1 M NaCl and 1 mM EDTA at 25 °C. The final concentration of PRI was 7.5 nM. After 15 s, a 12-μL aliquot of this mixture was assayed for activity toward 100 μM CpA in 600 μL of the same buffer, as described above. Incubation of PRI with the RNase A/angiogenin/2′-CMP mixture for periods longer than 15 s and up to 5 min resulted in the same amount of activity. In control assays, both angiogenin and PRI were omitted.

Results

Dissociation Rate Constant of RNase A·PRI. The rate constant of dissociation of the RNase A·PRI complex was determined by first forming the complex, then adding a scavenger for free PRI, and subsequently assaying for free RNase A released from the complex as a function of time. Free RNase A was quantitated either by cation-exchange HPLC or by enzymatic assay. The time courses for the appearance of free RNase A as determined by the two methods were quite similar (Figure 1). In both instances, a small amount of free RNase A, 4.2% of the total by HPLC and 14% by enzymatic assay, was released within several minutes after addition of scavenger while the remainder was released more slowly. Only the slow phase was used to calculate the dissociation rate constant of the complex. Concentrations of free RNase A in control samples lacking scavenger and both PRI and scavenger were 0% and ≥95%, respectively, of that of the total RNase A over the same period of time.

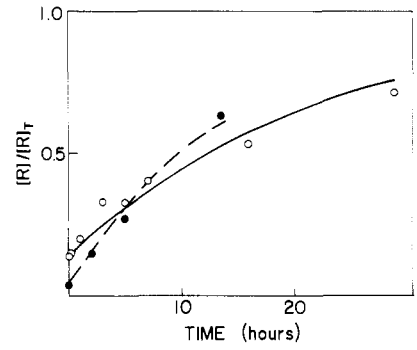


FIGURE 1: Dissociation kinetics of RNase A·PRI complex. RNase A was preincubated with 1.5 equiv of PRI for 20 min at 25 °C. Release of free RNase A, R, was followed by HPLC (●) or activity toward CpG (○) after addition of either 100 equiv of angiogenin (●) or 20 equiv of HT-RNase (○). $[R]_T$ is the total RNase A concentration. The buffer was 0.1 M Mes, pH 6, containing 0.1 M NaCl, 1 mM EDTA, and 5 mM DTT. The lines are calculated from the equation $[R] = [R]_T - ([R]_T - [R]_o)e^{-k_d t}$, where $[R]_o$ is the free RNase A concentration at the first time point after addition of scavenger and k_d is the dissociation rate constant of the RNase A·PRI complex. The values of k_d, 1.8×10^{-5} s^{-1} (– –) or 1.2×10^{-5} s^{-1} (—), are from plots of $-\ln\{([R]_T - [R])/([R]_T - [R]_o)\}$ vs t.

The concentrations of scavenger employed in these experiments were sufficiently high to ensure that concentrations of the free RNase A approached that of the total as $t \rightarrow \infty$. Under these conditions, the dissociation of the RNase A·PRI complex follows first-order kinetics for at least 60% of the reaction (Figure 1). The dissociation rate constant of the complex is 1.8×10^{-5} s^{-1} ($t_{1/2}$ = 11 h) and 1.2×10^{-5} s^{-1} ($t_{1/2}$ = 16 h) on the basis of HPLC and enzymatic activity, respectively.

Dissociation Rate Constant of Angiogenin·PRI. The rate constant of dissociation of the angiogenin·PRI complex was determined in an analogous manner. RNase A was used as the scavenger in 250-fold excess over angiogenin. Free angiogenin was quantitated by cation-exchange HPLC. In the absence of scavenger, there is no free angiogenin released from the complex even after 58 days of incubation. In its presence, a small amount of free angiogenin, 2.1% of the total, appears within several minutes, followed by a much slower release of the remainder over the course of ~45 days (Figure 2). Again, the dissociation rate constant was determined from this slow process.

During the course of the experiment, an apparent equilibrium is reached where some of the angiogenin remains bound to PRI. Thus, after 58 days, the normalized concentration of free angiogenin[2] is 80% of the total angiogenin concentration. Another sample in which PRI was added last to a mixture of angiogenin and RNase A also reaches the same

[2] During the several weeks required to observe appreciable dissociation of the complex, there was a gradual loss of ~40% of the free angiogenin in a control sample lacking PRI. To correct for the loss of free angiogenin in other samples, all free angiogenin concentrations were normalized to that present in this mixture. This treatment is valid if the rate of loss of free angiogenin approximates that of the angiogenin·PRI complex. The following observations suggest that this is indeed the case. Addition of 1 mM pHMB, which dissociates the angiogenin·PRI complex (Shapiro & Vallee, 1987), to the control sample containing only the angiogenin·PRI complex after 58 days released 41% of the total angiogenin. At this time, 61% of the total angiogenin was present in the control sample from which PRI was omitted. Thus, the rate of loss of angiogenin free in solution and complexed with PRI was similar.

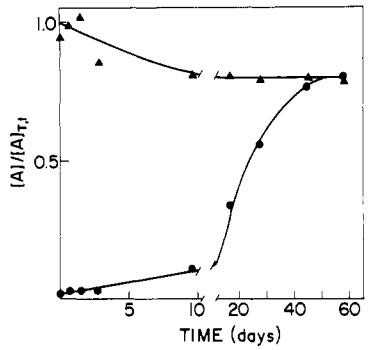


FIGURE 2: Dissociation kinetics of angiogenin·PRI complex. Angiogenin was preincubated with 1.5 equiv of PRI for 20 min at 25 °C. Release of free angiogenin, A, was determined by HPLC after addition of 250 equiv of RNase A (●). A second sample contained identical components except that PRI was added last (▲). $[A]_{T,t}$ is the angiogenin concentration measured in the control sample lacking PRI assayed at time t. The buffer was 0.1 M Mes, pH 6, containing 0.1 M NaCl, 1 mM EDTA, and 120 μM DTT. The line showing the release of free angiogenin during the first 10 days is calculated from the equation $[A] = [A]_{eq} - ([A]_{eq} - [A]_o)e^{-k_d t}$, where $[A]_{eq}$ is the free angiogenin concentration at equilibrium, $[A]_o$ is that observed at the first time point after addition of scavenger, and k_d is the dissociation rate constant of the angiogenin·PRI complex. All concentrations are normalized. The value of k_d, 1.3×10^{-7} s^{-1}, is from a plot of $-\ln\{([A]_{eq} - [A])/([A]_{eq} - [A]_o)\}$ vs t over the same time period.

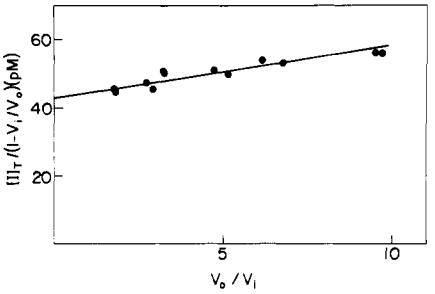


FIGURE 3: Henderson plot for inhibition of RNase A by PRI. RNase A (50 pM) was preincubated with PRI in 0.1 M Mes, pH 6, containing 0.1 M NaCl, 1 mM EDTA, 2 mM DTT, and 0.01% HSA for 80 min at 25 °C followed by addition of CpA to a final concentration of 100 μM. Hydrolysis of CpA was followed spectrophotometrically. $[I]_T$ is the total PRI concentration, and v_i and v_o are the initial velocities in the presence and absence of inhibitor, respectively.

free angiogenin concentration (Figure 2). Consistent with an equilibrium situation, the addition of 1 mM pHMB to the latter sample at day 58 results in the release of an additional 8% of angiogenin.

Since the rate of appearance of free angiogenin increases significantly after 10 days of incubation, the release of free angiogenin during the first 10 days was analyzed as a first-order process. During this time, there is about an 8% dissociation of the angiogenin·PRI complex. Setting the apparent equilibrium concentration of free angiogenin at day 58 equal to that which is free at $t = \infty$, the calculated dissociation rate constant of the complex is 1.3×10^{-7} s^{-1} ($t_{1/2}$ = 62 days).

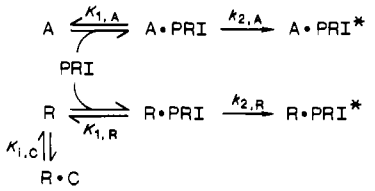
Henderson Plots for Inhibition of RNase A by PRI. The K_i value for the inhibition for RNase A by PRI can be calculated from the association and dissociation rate constants (see Discussion). The inhibition of RNase A by PRI was also analyzed by Henderson plots to obtain an independent esimate of the K_i value and to more readily examine the effects of pH and NaCl concentration on it. RNase A was preincubated with PRI before addition of 100 μM substrate, a concentration below the reported K_m, 320 μM (Witzel, 1963), so that the slope of the plot should approximate K_i. In 0.1 M NaCl at pH 6, the plot is almost flat (Figure 3), establishing an upper limit of 1.5 pM for the K_i value (see Discussion).

Table I: Effect of pH and Sodium Chloride Concentration on Inhibition of RNase A by PRI[a]

$[RNase A]_T$ (pM)	pH	[NaCl] (M)	K_i (pM)
50	5	0.1	19
50	6	0.1	<1.5
50	7	0.1	<0.9
200	8	0.1	<3.0
600	9	0.1	<4.0
100	6	0	<1.8
100	6	0.5	39
200	6	1.0	950

[a] Assays were performed as described under Experimental Procedures. $[RNase A]_T$ is the total RNase A concentration. K_i values were obtained from Henderson plots. 200 μM CpA was used at pH 7, 500 μM CpA at pH 8 and pH 9, and 100 μM CpA at all other conditions. At all pH values and NaCl concentrations, RNase A was preincubated with PRI before addition of substrate, and substrate concentrations were well below K_m.

Scheme I



Scheme II

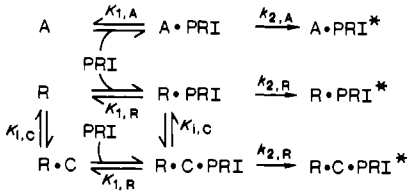


Table I shows the effects of pH and NaCl concentration on this value. At pH 7–9, the measured values similarly only set upper limits but do not vary significantly. Only at pH 5 is the K_i value substantially higher, 19 pM. Sodium chloride concentration has a marked effect. In 0.5 and 1 M NaCl, and K_i values are 39 and 950 pM, respectively.

Mode of Inhibition. The effect of a competitive inhibitor of RNase A, 2′-CMP, on the association rate of PRI with RNase A was investigated to determine the mode of inhibition. If PRI is a competitive inhibitor of RNase A, then increasing 2′-CMP concentrations should decrease the association rate of PRI with RNase A; if it is a noncompetitive inhibitor, then the association rate should be independent of 2′-CMP concentration.

The procedure employed for examining the association rate was similar to that used to determine the apparent second-order rate constant of the association of PRI with RNase A (Lee et al., 1989). In the present case, RNase A, angiogenin, and 2′-CMP were mixed, PRI was added, and the concentration of free RNase A was determined by enzymatic assay. Under the conditions of the experiment, angiogenin neither binds 2′-CMP nor cleaves the substrate employed, CpA (F. Lee and R. Shapiro, unpublished experiments; Shapiro et al., 1986a). If PRI binds to RNase A by the two-step mechanism observed for PRI and angiogenin (Lee et al., 1989), then Schemes I and II represent competitive and noncompetitive inhibition, respectively. Since k_{-2} is extremely low, it can be neglected for this experiment. In Schemes I and II, A is angiogenin, R is RNase A, and C is 2′-CMP.

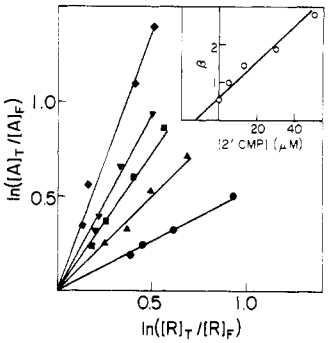


FIGURE 4: Determination of mode of inhibition of RNase A by PRI. RNase A (7.5 nM) was mixed at 25 °C with 0.8–4 equiv of angiogenin and 0 (●), 5 (▲), 12.5 (■), 30 (▼), or 50 μM (♦) 2′-CMP followed by addition of 1 equiv of PRI. Buffer was 0.1 M Mes, pH 6, containing 0.1 M NaCl and 1 mM EDTA. After 15 s, an aliquot was assayed for free RNase A by hydrolytic activity toward CpA. [A] and [R] are the angiogenin and RNase A concentrations, respectively. The subscripts T and F denote total and free concentrations, respectively. Inset: Effect of 2′-CMP concentration on the rate of association of RNase A with PRI. β is defined in the text. The line is obtained from linear regression of the data by using a weighting factor of $(1/\beta)^2$.

In this experiment, [PRI] is 7.5 nM, 70-fold below $K_{1,A}$, 530 nM, and also much less than $K_{1,R}$ (Lee et al., 1989). Under these conditions, it can be shown that

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

where the subscripts T and F denote total and free concentrations, respectively. γ equals $1 + [C]/K_{i,C}$ in Scheme I and 1 in Scheme II. 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 $k_{2,R}/K_{1,R}$.

The value of $[R]_F$ is determined directly from the enzymatic assay, and $[A]_F$ is then calculated. Figure 4 shows the results. The values of β at different 2′-CMP concentrations are shown in the inset. Since this term varies with 2′-CMP concentration, γ is 2′-CMP dependent, eliminating Scheme II. Scheme I predicts that the value of β will observed, and the absolute increasing 2′-CMP concentration and that the absolute value of the x intercept in this plot will equal $K_{i,C}$. A linear increase in the value of β is observed, and the absolute value of the x intercept, 12 μM, is in good agreement with that of 7 μM obtained for $K_{i,C}$ by Anderson et al. (1968). Thus, the data are consistent with a competitive mode of inhibition of RNase A by PRI.

Discussion

The binding of an RNase inhibitor, PRI, to angiogenin is an intriguing and functionally important aspect of the angiogenin/RNase A homology (Shapiro & Vallee, 1987). The determination of the K_i value for the binding of PRI to angiogenin is fundamental to an understanding of this interaction.

The K_i value can be determined on the basis of the two-step mechanism (Lee et al., 1989):

$$K_i = \frac{K_1 k_{-2}}{k_{-2} + k_2}$$

Since $k_2 \gg k_{-2}$, $K_i \simeq K_1 k_{-2}/k_2$. The values of K_1 and k_2 have been determined previously (Lee et al., 1989). The value of k_{-2} can be approximated closely by the dissociation rate constant of the EI* complex obtained here.[3] Thus, the K_i value for the inhibition of angiogenin by PRI is 0.71 fM (Table II) while that for RNase A, 44 fM, is about 60-fold higher. Both are extremely low. In fact, the dissociation constant for angiogenin and PRI is among the lowest reported for the interaction of two proteins.

Table II: Kinetic Parameters of Inhibition of Angiogenin and RNase A by PRI[a]

enzyme	K_1 (M)	k_2 (s^{-1})	k_2/K_1 (M^{-1} s^{-1})	k_{-2} (s^{-1})	K_i (M)
angiogenin	5.3×10^{-7}	97	1.8×10^8	1.3×10^{-7}	7.1×10^{-16}
RNase A	ND[b]		ND[b]	3.4×10^{8}[c]	1.5×10^{-5}	4.4×10^{-14}

[a] Conditions are 0.1 M Mes, pH 6, 0.1 M NaCl, 1 mM EDTA, and 25 °C. K_1, k_2, and k_{-2} correspond to the two-step mechanism described in the preceding paper (Lee et al., 1989). K_1, k_2, and k_2/K_1 were obtained from the preceding paper. [b] Not determined. [c] This value equals k_2/K_1 if the two-step mechanism for the inhibition of angiogenin by PRI holds for RNase A. If a one-step mechanism applies, it equals the second-order rate constant of association of PRI with RNase A. K_i for RNase A is the same for both mechanisms.

The dissociation rate constant of the angiogenin·PRI complex is a critical parameter of the inhibition, and there are two aspects of the experiment which bear comment. First, the dissociation of the angiogenin·PRI complex does not follow first-order kinetics over the full time course of this experiment. Rather, the rate of appearance of free angiogenin increases significantly after the first 10 days of incubation. This cannot be attributed to complete inactivation of inhibitor as a function of time: there was no free angiogenin in a control sample containing the angiogenin·PRI complex but no PRI scavenger over the whole course of the experiment.

It would be more reasonable to assume that the strength of the angiogenin·PRI interaction decreases with time, and there could be several possible causes. The sulfhydryl groups of PRI are essential for its activity and could be prime candidates for oxidation and, hence, inactivation (Blackburn & Moore, 1982). Deamidation could also contribute. Certainly these or other chemical changes could account for alterations in the kinetic parameters of inhibition by PRI over the long course of the experiment.

Second, an apparent equilibrium is reached at the end of the experiment where some angiogenin remains bound to PRI despite the 250-fold excess of RNase A over angiogenin. An independent estimate of the K_i value for the binding of PRI to angiogenin can be made:

$$K_{i,A} = \frac{K_{i,R}[A]_F[R\cdot PRI^*]}{[R]_F[A\cdot PRI^*]}$$

where $K_{i,A}$ and $K_{i,R}$ are the inhibition constants for PRI binding to angiogenin and RNase A, respectively, $[A]_F$ and $[R]_F$ are the free angiogenin and RNase A concentrations at equilib-

[3] Under the two-step mechanism (Lee et al., 1989), the experimentally determined rate constant for the dissociation of the EI* complex, k_d, equals

$$k_d = k_{-2} + \frac{k_2 k_1 [PRI]_F}{k_{-1} + k_1[PRI]_F}$$

where $[PRI]_F$ is the free PRI concentration. If the free enzyme concentration approaches the total enzyme concentration as $t \rightarrow \infty$, then $k_d \simeq k_{-2}$. This was the case with the experiments examining the RNase A·PRI complex. If, on the other hand, the free enzyme concentration does not approach the total enzyme concentration when $t \rightarrow \infty$, as was the case with the experiment examining the angiogenin·PRI complex, then $k_d > k_{-2}$. A conservative estimate based on the assumption that $[PRI]_F$ is determined solely by its equilibrium with [R·PRI] is that k_{-2} is minimally 62% of k_d.

rium, respectively, and [A·PRI*] and [R·PRI*] are the concentrations of the PRI complexes with angiogenin and RNase A at equilibrium, respectively. By using the value of 44 fM calculated for $K_{i,R}$ and determining all other concentrations from the amount of free angiogenin measured at the apparent equilibrium (Figure 2, day 58), a value of 0.92 fM for $K_{i,A}$ is obtained. This is in good agreement with the $K_{i,A}$ value calculated from the association and dissociation rate constants, 0.71 fM. Thus, it would seem that the apparent equilibrium is probably a true equilibrium.

The calculated dissociation rate constant of the angiogenin·PRI complex is not affected markedly by small uncertainties in the free angiogenin concentration chosen when $t = \infty$. For example, if the concentration of free angiogenin at $t = \infty$ was half the apparent equilibrium concentration observed at day 58, the dissociation rate constant would increase only by a factor of 2.

The inhibition of RNase A by PRI was analyzed by Henderson plots to determine the effects of pH and NaCl concentration on it (Figure 3; Table I). Unlike standard kinetic plots, this analysis takes inhibitor depletion into account and has been employed for the analysis of tight-binding inhibitors (Henderson, 1972). However, the K_i value for the inhibition of RNase A obtained from the Henderson plot at pH 6, 1.5 pM (Figure 3), is more than 1 order of magnitude higher than that calculated, 44 fM. This suggests that the value obtained from the Henderson plot represents only an upper limit for the true K_i value. This may stem from the fact that Henderson plots do not allow accurate K_i determinations at high $[E]_T/K_i$ ratios (Henderson, 1972). In this case, the RNase A concentration employed, 50 pM, is 33 times the K_i value obtained from the Henderson plot and more than 1000 times the K_i value calculated from its association and dissociation rate constants. Since this reasoning also applies to the Henderson plots obtained at pH 7–9, the values calculated from them most likely are only upper limits as well (Table I). It follows that there could be a greater pH dependence than that suggested by the Henderson plots.

On the other hand, the K_i values obtained by Henderson plot at 0.5 and 1.0 M NaCl likely represent true K_i values because in these assays $0.1 < [E]_T/K_i < 10$ (Table I). The K_i value changes 20 000-fold from 0.1 to 1.0 M NaCl, suggesting that ionic interactions play a significant role in the binding of PRI to RNase A. This provides a rationale for the use of 3 M NaCl to elute PRI from its affinity column, RNase A–Sepharose (Blackburn et al., 1977). It has been shown previously that changes in sodium chloride concentration affect the association rate of PRI with angiogenin (Lee et al., 1989).

The K_i value for the binding of PRI to RNase A determined here is more than 3 orders of magnitude lower than values previously reported for PRI and related inhibitors (Blackburn et al., 1977; Burton et al., 1980; Turner et al., 1983). This cannot be attributed solely to differences in pH or ionic strength, since the K_i values obtained here from Henderson plots under comparable conditions are all much lower than those reported previously. In some of the studies (Blackburn et al., 1977; Burton et al., 1980), the use of Lineweaver–Burk and Dixon plots could be one possible cause of the discrepancy, since they do not take inhibitor depletion into account (Morrison, 1969). In two of the cases, the fact that the inhibitor was isolated from a different source could be another possible cause (Burton et al., 1980; Turner et al., 1983).

Earlier studies suggested that PRI and ribonuclease inhibitors from other sources are noncompetitive inhibitors of RNase A (Bishay & Nicholls, 1973; Bartholeyns & Baudhuin, 1977; Blackburn et al., 1977; Burton et al., 1980). However, these studies relied on Lineweaver–Burk and Dixon plots, in which tight-binding inhibitors can appear to act in a noncompetitive manner, regardless of the actual inhibition mode (Morrison, 1969).

In principle, the inhibition mode for RNase A is ascertainable by examining the effect of substrate concentration on the inhibition by PRI under steady-state conditions with Henderson plots. However, steady state is not reached during the time course of the assays due to the slow rate of dissociation. Thus, the inhibition mode was determined by examining the effect of a competitive inhibitor of RNase A on the association rate of PRI with RNase A. The data rule out a pure noncompetitive mode of inhibition of RNase A by PRI (Figure 4) and are consistent with a competitive mode (Scheme I).[4] Many of the residues in the proposed contact region of RNase A with PRI (Blackburn & Moore, 1982) are conserved in angiogenin [see Shapiro and Vallee (1987)], supporting the view that PRI also inhibits angiogenin competitively.

PRI is about a 60-fold more potent inhibitor of angiogenin than of RNase A. This, in part, may reflect the greater preponderance of basic residues in angiogenin (Strydom et al., 1985) since PRI is acidic (Blackburn et al., 1977). The sodium chloride concentration dependence of the K_i value for PRI binding to RNase A indicates the importance of ionic interactions. It might also be noted that both PRI and angiogenin are human proteins whereas RNase A is a bovine protein.

The potent inhibition of angiogenin by PRI strongly suggests that PRI regulates its activity. One possibility is that the inhibitor may serve to terminate the action of angiogenin. In this respect, the extremely low K_i value for the binding of PRI to angiogenin is appropriate for the inhibition of a protein capable of inducing neovascularization at femtomolar levels (Fett et al., 1985). Alternatively, angiogenin could also be present as an enzyme·PRI complex which releases free angiogenin when the inhibitor is inactivated.

The tight-binding inhibition of angiogenin by PRI opens new approaches to antiangiogenesis. Structure–function studies on the angiogenin·PRI interaction should identify areas in both molecules critical for binding and aid in the design of novel angiogenin inhibitors. In this regard, the primary structure of PRI will be of particular value and is the subject of a future paper.

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[4] A mixed mode of inhibition is not ruled out. However, the data indicate that PRI binds at least 10-fold more rapidly to R than to R·C.

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Concerted Two-Dimensional NMR Approaches to Hydrogen-1, Carbon-13, and Nitrogen-15 Resonance Assignments in Proteins†

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ABSTRACT: When used in concert, one-bond carbon–carbon correlations, one-bond and multiple-bond proton–carbon correlations, and multiple-bond proton–nitrogen correlations, derived from two-dimensional (2D) NMR spectra of isotopically enriched proteins, provide a reliable method of assigning proton, carbon, and nitrogen resonances. In contrast to procedures that simply extend proton assignments to carbon or nitrogen resonances, this technique assigns proton, carbon, and nitrogen resonances coordinately on the basis of their integrated coupling networks. Redundant spin coupling pathways provide ways of resolving overlaps frequently encountered in homonuclear ^{1}H 2D NMR spectra and facilitate the elucidation of complex proton spin systems. Carbon–carbon and proton–carbon couplings can be used to bridge the aromatic and aliphatic parts of proton spin systems; this avoids possible ambiguities that may result from the use of nuclear Overhauser effects to assign aromatic amino acid signals. The technique is illustrated for *Anabaena* 7120 flavodoxin and cytochrome *c*-553, both uniformly enriched with carbon-13 (26%) or nitrogen-15 (98%).

Nuclear magnetic resonance is the only serious competitor to X-ray crystallography for detailed structural studies of proteins. NMR[1] structures determined in solution are of low resolution, but they may better represent the physiological conformations or the dynamic properties of proteins than structures derived from crystals. In addition, NMR can be used to investigate conformational equilibria, interactions of proteins with other proteins or small molecules, and reaction pathways involving covalent protein modification.

The key step in protein NMR investigations is the assignment of proton, carbon, and nitrogen resonances to specific amino acids in the protein. The most successful approach in small proteins has been to first assign the proton spin systems by using a combination of two-dimensional (2D) homonuclear proton NMR experiments (Billeter, 1982; Wüthrich et al., 1982; Wüthrich, 1986), such as COSY (Nagayama et al., 1980), NOESY (Kumar et al., 1980), RELAY (Bax & Drobny, 1985), and HOHAHA (Davis & Bax, 1985). Proton assignments are then extended to carbon or nitrogen resonances by using heteronuclear correlation experiments (Chan & Markley, 1982; Kojiro & Markley, 1983; Ortiz-Polo et al., 1986; Glushka & Cowburn, 1987; Sklenar & Bax, 1987). The complexity and overlaps in homonuclear ^{1}H{^{1}H} 2D spectra, however, make this approach difficult with larger proteins (M_r >10 000).

Heteronuclei can be used to resolve overlaps in 2D proton spectra. Examples include spectral editing techniques for

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[1] Abbreviations: 2D, two dimensional; COSY, correlated spectroscopy; DQC, double quantum correlation; HOHAHA, homonuclear Hartmann-Hahn; INADEQUATE, incredible natural abundance double quantum transfer; MBC, multiple-bond correlation; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser spectroscopy; RELAY, relayed correlated spectroscopy; SBC, single-bond correlation; TMS, tetramethylsilane; TSP, (trimethylsilyl)propionic acid.

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