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Interaction of Human Placental Ribonuclease with Placental Ribonuclease Inhibitor†

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ABSTRACT: The interactions of human placental ribonuclease inhibitor (PRI) with bovine pancreatic ribonuclease (RNase) A and human angiogenin, a plasma protein that induces blood vessel formation, have been characterized in detail in earlier studies. However, studies on the interaction of PRI with the RNase(s) indigenous to placenta have not been performed previously, nor have any placental RNases been identified. In the present work, the major human placental RNase (PR) was purified to homogeneity by a five-step procedure and was obtained in a yield of 110 μg/kg of tissue. The placental content of angiogenin was also examined and was found to be at least 10-fold lower than that of PR. On the basis of its amino acid composition, amino-terminal sequence, and catalytic properties, PR appears to be identical with an RNase previously isolated from eosinophils (eosinophil-derived neurotoxin), liver, and urine. The apparent second-order rate constant of association for the PR·PRI complex, measured by examining the competition between PR and angiogenin for PRI, is 1.9×10^8 M^{-1} s^{-1}. The rate constant for dissociation of the complex, determined by HPLC measurement of the rate of release of PR from its complex with PRI in the presence of a scavenger for free PRI, is 1.8×10^{-7} s^{-1}. Thus the K_i value for the PR·PRI complex is 9×10^{-16} M, similar to that obtained with angiogenin, and 40-fold lower than that measured with RNase A. Complex formation causes a small red shift in the protein fluorescence emission spectrum, with no significant change in overall intensity. The fluorescence quantum yield of PR and the Stern-Volmer constant for fluorescence quenching by acrylamide are both high, possibly due to the presence of an unusual posttranslationally modified tryptophan residue at position 7 in the primary sequence.

Despite several decades of study [see Levy and Karpetsky (1981)], human ribonucleases (RNases)[1] have only recently begun to be characterized as molecular entities. Five structurally and functionally distinct species have now been identified, including pancreatic RNase (Weickmann et al., 1981; Beintema et al., 1984), the "nonsecretory" RNase from liver, urine, and eosinophils (Cranston et al., 1980; Gleich et al., 1986; Beintema et al., 1988a; Sorrentino et al., 1988), and three additional forms found in blood (see Discussion). These RNases are closely related: their amino acid sequences are homologous and all cleave preferentially on the 3′ side of pyrimidines, albeit at different rates and with somewhat different specificities.

Contrasting with the considerable amount of knowledge obtained concerning the primary structures and, in some instances, functional properties of these enzymes is the relative scarcity of information available concerning their distribution throughout the body. Thus, blood, urine, pancreas, and liver remain the only sources from which human RNases have been purified to homogeneity and definitively identified. The nature of the RNases in other tissues has been explored thus far

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[1] Abbreviations: RNase(s), ribonuclease(s); RNase A, bovine pancreatic ribonuclease A; PRI, placental ribonuclease inhibitor; PR, placental ribonuclease; TSE, 20 mM Tris, pH 7.5, containing 0.25 M sucrose and 1 mM EDTA; CM, carboxymethyl; C18, octadecylsilane; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Mes, 2-morpholinoethanesulfonic acid; CpA, cytidylyl-3′,5′-adenosine; UpA, uridylyl-3′,5′-adenosine; HSA, human serum albumin; pHMB, *p*-(hydroxymercuri)benzoate; EDN, eosinophil-derived neurotoxin; PTH, phenylthiohydantoin; ECP, eosinophil cationic protein.

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primarily by indirect means, involving the examination of RNase contents of crude extracts by immunological (Neuwelt et al., 1977, 1978; Weickmann & Glitz, 1982; Morita et al., 1986; Sorrentino et al., 1988) or enzymatic (Neuwelt et al., 1978; Weickmann & Glitz, 1982) assays. The results of these studies suggest that both the pancreatic- and liver-type enzymes are widely distributed, with the former predominating in pancreas, brain, kidney, and heart and the latter in liver, spleen, lung, and stomach.

In addition to RNases, mammalian tissues contain cytoplasmic RNase inhibitor proteins that bind to both the pancreatic- and liver-type enzymes (Roth, 1967; Blackburn & Moore, 1982). Placenta is a rich source of such an inhibitor (Blackburn et al., 1977), and the interactions of human placental RNase inhibitor (PRI) with bovine pancreatic RNase A and human angiogenin, an RNase homologue, have been examined in detail (Blackburn & Moore, 1982; Lee et al., 1989a,b). However, studies on the interaction of PRI with human tissue RNases, in particular, with the RNase(s) indigenous to placenta, have not been performed previously.

In the present report, we describe the isolation and characterization of the major RNase from human placenta. This enzyme has catalytic and structural properties distinct from those of pancreatic RNase and appears to be identical with the predominant liver RNase (Sorrentino et al., 1988). The dissociation constant for its complex with PRI, calculated from the rate constants for association and dissociation, is extremely low, 9×10^{-16} M. This value is about 40-fold lower than that measured with RNase A and similar to that obtained with angiogenin (Lee et al., 1989b). Changes in protein fluorescence accompanying placental RNase (PR)·PRI complex formation have also been examined.

Experimental Procedures

Materials. Recombinant human angiogenin was prepared as described (Kurachi et al., 1988) and was quantitated by amino acid analysis. UTP–agarose was prepared as described by Lamed et al. (1973). Sources of all other materials employed have been listed elsewhere (Shapiro et al., 1986a,b, 1987).

Purification of RNase from Human Placenta. Placentas were obtained within 2 h after delivery, washed with TSE (20 mM Tris, 0.25 M sucrose, 1 mM EDTA, pH 7.5), and cut into small pieces. These pieces were then blotted on paper towels in order to remove as much blood as possible and stored at −70 °C. After thawing, tissue (600–900 g) was homogenized in TSE (2 mL/g) in a blender. All subsequent operations were performed at 4 °C unless indicated otherwise. Cell debris was removed by centrifugation for 30 min at 16000*g*. The supernatant was brought to pH 3 with glacial acetic acid and frozen at −20 °C. After 5 days, the homogenate was thawed and centrifuged as above. The supernatant was then filtered through Whatman 934-AH glass microfiber paper and dialyzed vs 20 mM sodium phosphate, pH 6.0. The retentate was brought to pH 6.0 with sodium hydroxide, centrifuged and filtered as above, and loaded onto a 2.5 × 35 cm column of CM-52 cation-exchange resin that had been equilibrated with 20 mM sodium phosphate, pH 6.0. Elution was achieved with a 4-L gradient from 0 to 1 M NaCl in the same buffer at a flow rate of 150 mL/h. Fractions from the major peak of RNase activity were pooled and dialyzed into 0.1 M Tris, pH 7.5, in an Amicon ultrafiltration device equipped with a YM 5 membrane. The sample (5 mL) was then centrifuged (15600*g*, 15 min) and the supernatant loaded onto a 1.6 × 98 cm column of Sephadex G-50 (superfine) that had been equilibrated with 0.1 M Tris, pH 7.5, at a flow rate of 5 mL/h. Fractions containing RNase activity were pooled and dialyzed into 25 mM piperazine, pH 5.3, in an Amicon ultrafiltration device. This material was then applied to a 0.8 × 5 cm column of UTP-agarose at a flow rate of 5 mL/h. After the column was washed with the piperazine buffer, RNase was eluted with 0.25 M sodium phosphate, pH 5.45 (Smith et al., 1978). Fractions containing RNase activity were pooled, dialyzed into 20 mM sodium acetate, pH 5.5, and then applied to a Mono S cation-exchange column (5 × 50 mm; Pharmacia) equilibrated in the same buffer. Elution was achieved with a 50-min gradient from 0 to 0.4 M NaCl in the acetate buffer at 0.8 mL/min at ambient temperature. Material in the peak containing RNase activity, eluting at 36 min, was then chromatographed on a Synchropak C18 HPLC column (Synchrom Inc., Linden, IN) (10-μm particle size, 4.6 × 250 mm) as described in the legend for Figure 2. The final preparation was quantitated by amino acid analysis.

Isolation of Angiogenin from Human Placenta. Angiogenin was isolated from placental homogenate by a modification of procedures previously employed for its purification from HT-29 cell conditioned medium (Fett et al., 1985) and plasma (Shapiro et al., 1987). Homogenate was acidified, frozen and thawed, concentrated, dialyzed, and chromatographed on CM-52 cation-exchange resin (50 mL of resin/kg of tissue) essentially as described (Fett et al., 1985). Material that binds to the CM-52 resin in 0.1 M sodium phosphate, pH 6.6, and elutes with 1 M NaCl was dialyzed vs water, lyophilized, and then chromatographed on phenyl and C18 reversed-phase HPLC columns (Shapiro et al., 1987).

Physicochemical Characterization. SDS–PAGE was performed by using 15% gels as described by Laemmli (1970). Gels were silver-stained with a kit from ICN. Amino acid analyses (PicoTag method; Waters Associates) and N-terminal sequence analyses were performed as described (Strydom et al., 1985). UV spectra were recorded on a Varian 219 spectrophotometer.

RNase Assays. Activity toward high molecular weight wheat germ RNA was measured by a modification of the precipitation assay of Blank and Dekker (1981). Reaction mixtures containing 33 mM Hepes, pH 7.3, 33 mM NaCl, 0.6 mg of RNA, 30 μg of HSA, and sample (diluted in 0.01% HSA) in a volume of 300 μL were incubated at 37 °C for 30 min. The reaction was stopped by addition of 700 μL of ice-cold 3.4% perchloric acid, and after 10 min on ice the mixtures were centrifuged at 15600*g* for 10 min at 4 °C. The absorbance of the supernatants was measured at 260 nm in a 1-cm cuvette. This assay was used throughout the isolation procedures. Changes in absorbance at 260 nm were linearly dependent on RNase concentration up to a value of ~2.0. Activity toward polyhomoribonucleotides was determined by the method of Zimmerman and Sandeen (1965) as described (Shapiro et al., 1986b).

Activity toward dinucleotides was measured either by continuous spectrophotometric assay (Witzel & Barnard, 1962) or by HPLC quantitation of products and starting material (Shapiro et al., 1986a,b). The spectrophotometric method was employed when CpA or UpA was the substrate; the HPLC method was used with all other dinucleotides. Unless specific otherwise, assays were performed with 0.1 mM substrate in 33 mM Mes, pH 6.0, containing 33 mM NaCl at 25 °C.

Determination of Nucleotide at 3′ Termini of PR Digest of RNA. A modification of previous methods (Farkas & Marks, 1968; Shapiro et al., 1986a) was employed. Wheat germ RNA was incubated with 0.1 nM PR for 40 min at 37

°C as described above. Perchloric acid soluble products were treated with alkaline phosphatase and base-hydrolyzed as described (Shapiro et al., 1986a). This procedure should convert to free nucleoside any nucleotide donating a 3′-phosphate to a cleaved bond, while the remainder are converted to a mixture of 2′- and 3′-nucleotides. The distribution of nucleosides and nucleotides was determined by C18 HPLC (Shapiro et al., 1986a).

Fluorescence Measurements. Fluorescence measurements were performed at 25 °C with a Perkin-Elmer Model MPF-3 fluorescence spectrophotometer equipped with a Model 150 xenon lamp and a Hitachi Model QPD33 recorder. Emission spectra and quenching measurements were obtained as described by Lee et al. (1989a). Fluorescence quenching by acrylamide was analyzed by Stern–Volmer plots of F_0/F vs acrylamide concentration, where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively. The Stern–Volmer quenching constant, K_{sv}, was obtained from the initial slope of these plots. Samples (0.08 μM PR or 2 μM indole) were in 10 mM Tris, pH 7.5. Excitation was at 295 nm in order to minimize any contribution of tyrosines in PR. Emission was monitored at 340 nm for PR and at 348 nm for indole.

Interaction of PR with PRI. The apparent second-order rate constant for association of PRI with PR was determined by examining the competition between PR and human angiogenin for PRI. PR (4.2 nM) was mixed with 0.6–1.4 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 4.2 nM. After 15 s, the activity of free PR was measured by adding the dinucleotide CpA (final concentration 100 μM) and continuously monitoring the decrease in absorbance at 286 nm. Angiogenin does not cleave CpA at a detectable rate. No significant dissociation of angiogenin (Lee et al., 1989b) or PR (see below) from their complexes with PRI occurs during the course of these assays. The apparent second-order rate constant of association was then calculated by using the equation

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

where $k_{a,A}$ is the apparent second-order rate constant for association of PRI with angiogenin (1.8×10^8 M^{-1} s^{-1}) and $[R]_T$, $[R]_F$, $[A]_T$, and $[A]_F$ are the total PR, free PR, total angiogenin, and free angiogenin concentrations, respectively (Lee et al., 1989a). The PRI stock solution was quantitated by a titration plot that measures inhibition of PR in this assay system, assuming a 1:1 stoichiometry. This plot was linear, indicating that the PRI concentration was well above K_i.

The rate constant for dissociation of the PR·PRI complex was measured by a modification of a previously described procedure (Lee et al., 1989b). PR (50 nM) and PRI (75 nM) were incubated for 20 min at 25 °C in 0.1 M Mes, pH 6, containing 0.1 M NaCl and 10 μg/mL HSA. Angiogenin was then added (14 μM final concentration) as scavenger for any free PRI dissociating from the complex, and the mixture was incubated at 25 °C. At various times, aliquots were removed and assayed for PR activity by using the CpA assay described above.

Results

Purification of RNase from Human Placenta. Most of the RNase activity in placental homogenate is present in "latent" form, presumably complexed with PRI, as observed previously (Blackburn et al., 1977). Thus, incubation of an aliquot of homogenate with 1 mM pHMB, a treatment that inactivates both free and complexed PRI (Blackburn et al., 1977) and liberates any bound RNase, increased activity toward wheat germ RNA nearly 10-fold. A similar enhancement of activity was observed following acidification and freeze–thawing of the homogenate; addition of pHMB to an aliquot of the treated homogenate did not increase activity further.

Table I: Purification of Human Placental RNase

step	vol (mL)	protein[a] (total mg)	RNase activity[b] (total units)
extract	2000	22000	29200
acidification/ freeze–thawing	2050	16800	253000
CM-52	195	62.4	158000
Sephadex G-50	18.5	13.1	135000
UTP–agarose	8.1	0.77	93200
Mono S	3.2	0.28	75000
C18	3.2	0.10	70300

[a] Protein quantitations were obtained by amino acid analysis for the UTP–agarose, Mono S, and C18 samples and by the procedure of Bradford (1976) for the remainder. [b] RNase activity was determined with wheat germ RNA as substrate as described in the text. RNase A (125 pg) was included as a standard for each set of assays performed. One unit of activity is defined as the activity of 1 ng of RNase A.

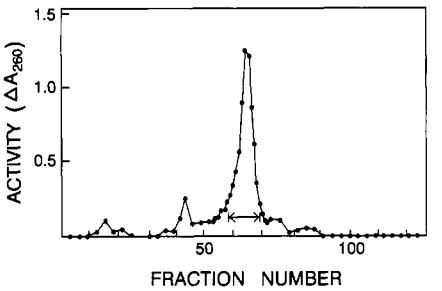

FIGURE 1: Chromatograph of placental homogenate on CM-52. Homogenate from 900 g of placenta, prepared for chromatography as described in the text, was applied to a 2.5 × 40 cm column of CM-52 that had been equilibrated with 20 mM sodium phosphate, pH 6.0. After it was loaded, the column was washed with 200 mL of equilibration buffer. Elution was achieved with a 4-L linear gradient of 0–1.0 M NaCl in the same buffer. Fifteen-milliliter fractions were collected and assayed for activity toward wheat germ RNA at pH 7.3 (see Experimental Procedures). The activities shown represent ΔA_{260} values obtained with 5 μL of a 20-fold diluted aliquot from each fraction. Less than 10% of the RNase activity loaded onto the column eluted in the drop-through fractions (not shown). Fractions 57–69 were pooled for further purification.

RNase was isolated from acidified/freeze–thawed homogenate by a five-step procedure summarized in Table I: CM-52 cation-exchange chromatography, gel filtration on Sephadex G-50, affinity chromatography on UTP–agarose, Mono S cation-exchange HPLC, and C18 HPLC (see Experimental Procedures). About 70% of the RNase activity eluted from CM-52 as a single major peak (Figure 1). Subsequent gel filtration, affinity chromatography, and Mono S HPLC revealed a single peak of activity in each case. C18 HPLC (Figure 2), however, yielded two peaks, at 26.4 and 28.0 min, which contained RNase activity. Both the specific activities and the amino acid compositions of material in the two peaks were indistinguishable. Only material from the second, larger peak was employed in subsequent studies. The final preparation—110 μg/kg of starting material—contained 28% of the RNase activity measured in the initial homogenate after acidification and freeze–thawing. Assuming that the activity in the treated homogenate was primarily due to the species subsequently purified, the overall extent of purification was about 60000-fold. The specific activity of this protein (PR) toward high molecular weight wheat germ RNA is 70% of that measured with RNase A under the standard assay conditions.

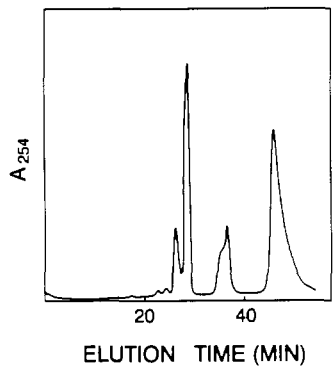

FIGURE 2: Chromatograph of Mono S purified placental RNase on a Synchropak C18 HPLC column. Full-scale absorbance is 0.045. Solvent A was 0.1% TFA in water. Solvent B was 0.08% TFA in a 3:2:2 2-propanol/acetonitrile/water mixture. Elution was achieved with a 90-min linear gradient from 25% to 75% solvent B at 0.8 mL/min. Fractions were collected at 1-min intervals and assayed for RNase activity as described in the text. The proteins eluting at 26.4 and 28.0 min accounted for >90% of the RNase activity loaded onto the column.

Purification of Angiogenin from Placenta. The purification procedures just described were designed for the isolation of acid-stable RNases that are active toward high molecular weight RNA. Angiogenin, a blood vessel inducing protein that has ribonucleolytic activity (Shapiro et al., 1986b) and binds tightly to PRI (Shapiro & Vallee, 1987), is relatively inactive toward this substrate and therefore would not have been detected on this basis. In order to examine the content of this protein in placenta, an alternative protocol was employed (see Experimental Procedures), based on the known chromatographic properties of the protein. This procedure yielded about 6 μg/kg of tissue of a protein having the same phenyl and C18 HPLC elution times as angiogenin. Its identity as angiogenin was then established by SDS–PAGE and amino acid analysis (not shown). Some of this protein may have derived from blood present in the placental homogenate rather than from the tissue itself: normal adult plasma contains ~400 μg/L angiogenin (K. A. Olson, personal communication), while levels in fetal blood have not been determined. Regardless of the ultimate origin of the angiogenin isolated from placenta, it appears to be at least 10-fold less abundant than PR, assuming that the yield of angiogenin during the purification is not significantly lower than that for PR. [The yield of angiogenin from plasma utilizing the same procedure is 20–40% (unpublished results).]

Physicochemical Characterization of PR. SDS–PAGE of the final C18 HPLC purified PR preparation revealed a single diffuse band of apparent molecular weight ~18000 that stains poorly with silver (not shown). The amino acid composition of this protein (Table II) is indistinguishable from those of human liver RNase (Sorrentino et al., 1988), RNase U_s from urine (Beintema et al., 1988a), and eosinophil-derived neurotoxin (EDN) (Rosenberg et al., 1989; Hamann et al., 1989). Automated Edman degradation of the amino-terminal 22 residues indicates a sequence identical with that previously reported for the liver, urine, and eosinophil RNases (Gleich et al., 1986; Beintema et al., 1988a; Sorrentino et al., 1988): Lys-Pro-Pro-Gln-Phe-Thr-X-Ala-Gln-Trp-Phe-Glu-Thr-Gln-His-Ile-X-Met-Thr-Ser-Gln-Gln. As with the previous isolates, no PTH-amino acid was observed for positions 7 and 17, suggesting that these residues have been modified posttranslationally.

Substrate Specificity of PR. The specificity of PR was investigated in order to more definitively establish its relationship to known RNases and to provide a basis for studying its interaction with PRI. Under the conditions employed, PR cleaves poly(U) ~3-fold faster than poly(C) and does not degrade either poly(A) or poly(G) at a detectable rate. The rates of poly(U) and poly(C) cleavage are 4- and 800-fold slower, respectively, than those obtained with RNase A.

Table II: Amino Acid Compositions of Human Placental and Liver RNase and RNase U_s

amino acid	placental RNase	liver RNase[a]	RNase U_s[b]
Asx	21.5	21.2	21
Glx	14.0	14.9	14
Ser	7.3	8.0	6
Gly	2.4	2.9	2
His	4.6	5.0	5
Arg	8.1	8.3	8
Thr	12.6	13.0	12
Ala	6.4	6.7	6
Pro	12.0	12.6	12
Tyr	4.0	4.2	4
Val	7.8	11.5	9
Met	4.0	3.4	4
Ile	5.6	6.8	7
Leu	4.8	5.5	5
Phe	5.1	5.4	5
Lys	4.1	4.6	4
Trp	1.0	nd[c]	1
Cys	nd	nd	8

[a] Calculated from mole percent values of Sorrentino et al. (1988) assuming 134 residues. [b] From sequence (Beintema et al., 1988). [c] Not determined.

Table III: Activities of Human PR and Bovine RNase A toward Dinucleotides

	k_{cat}/K_m[a] (M^{-1} s^{-1})	
dinucleotide	PR	RNase A
CpA	294000	4590000
UpA	144000	3230000
CpG	541	476000
UpG	133	141000
CpC	20	139000
CpU	11	24300
UpU	2.5	12200
UpC	<1.5	41300
all ApN′	<0.5	nd[b]
all GpN′	<0.5	nd

[a] Reaction conditions were 33 mM Mes and 33 mM NaCl, pH 6.0, 25 °C. The k_{cat}/K_m values with CpA and UpA were determined by spectrophotometric assay. With the other dinucleotides, k_{cat}/K_m values were measured by using an HPLC assay as described (Shapiro et al., 1986a). [b] Not determined.

Cleavage of dinucleotides was measured either by continuous spectrophotometric assay or by HPLC quantitation of substrate and products. The action of PR on only two dinucleotides—CpA and UpA—was amenable to study by the first method. Values for k_{cat}/K_m of 2.9×10^5 M^{-1} s^{-1} and 1.4×10^5 M^{-1} s^{-1} were determined for CpA and UpA, respectively. These values are 16- and 22-fold lower, respectively, than those measured with RNase A. A Lineweaver–Burk plot (not shown) with CpA as substrate yielded a K_m value of 3 mM and a k_{cat} value of 900 s^{-1}, compared to values of 0.5 mM and 2300 s^{-1} for RNase A.

Cleavage of the remaining 14 common dinucleotides (NpN′) was examined with an extremely sensitive HPLC assay (Table III). Conversion of all CpN′ and UpN′ dinucleotides except for UpC to free nucleoside N′ and C>p or U>p was observed. No reaction was detected with ApN′ and GpN′ dinucleotides. The k_{cat}/K_m values in Table III indicate that only CpA and UpA are cleaved at appreciable rates. Replacement of adenosine at position N′ by guanosine decreases the rate by about three orders of magnitude; with cytidine or uridine at

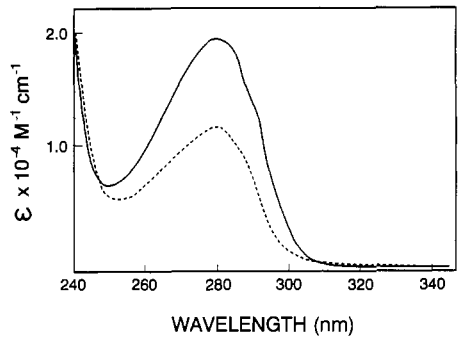

FIGURE 3: UV absorption spectra of PR (—) and angiogenin (---). Spectra were recorded on a Varian 219 spectrophotometer, and calculations were performed with an Apple program. Samples (200 μL) were in 20 mM Tris, pH 7.5. Quantitations (5.4 μM for PR, 7.7 μM for angiogenin) were obtained by duplicate amino acid analyses.

position N′, the reaction is slower by at least an additional order of magnitude.

Substrate specificity was also assessed by digesting wheat germ RNA with PR and then identifying the nucleotides present at the newly formed 3′ termini (see Experimental Procedures). Digestion was carried out until ~10% of the RNA became perchloric acid soluble so that the kinetic cleavage preferences, only apparent at early stages in the reaction, could be determined. The results are consistent with the pattern observed for dinucleotides, with cleavage occurring almost exclusively at CpN′ and UpN′ bonds, 27% and 24% of which were broken, respectively.

Interaction of PR with PRI: *(A) Inhibition of PR Activity.* The activity of 3 nM PR toward CpA is completely inhibited by addition of 1 molar equiv of PRI, indicating that the K_i value is well below 1 nM. This tightness of binding precludes the use of standard kinetic procedures for K_i determination (e.g., Henderson plots): such methods require the utilization of much lower enzyme concentrations so that inhibition is substoichiometric, and this cannot be achieved in any of the assay systems available. Alternative approaches for determining K_i values, based on measurements of the rate constants for association and dissociation of the PR·PRI complex, are described below.

(B) Effect of Complex Formation on Fluorescence. The association of PRI with angiogenin was examined previously by stopped-flow kinetic techniques, making use of a substantial tryptophan fluorescence increase during binding (Lee et al., 1989a). In order to determine whether a similar approach is feasible with PR, the UV absorbance and fluorescence properties of PR and the effects of complex formation on fluorescence were examined. The UV absorption spectrum of PR (Figure 3) reveals a maximum at 280 nm with a molar absorptivity of 19 500 M^{-1} cm^{-1}. This absorptivity is unusually high for a protein containing only the single tryptophan and four tyrosines indicated by amino acid analysis (Table II). Angiogenin, which also contains one tryptophan and four tyrosine residues, has a molar absorptivity of 12 500 M^{-1} cm^{-1} at 280 nm (Figure 3). The additional absorbance in PR appears to be largely contributed by a posttranslationally modified tryptophan residue at position 7 (see Discussion).

The fluorescence emission spectra of PR, PRI, and the PR·PRI complex are shown in Figure 4. All have maxima (335–340 nm) and shapes typical of tryptophan. The spectrum of PR is about 75% as intense as that of PRI, which contains six tryptophans (Lee et al., 1988), and five times more intense than that of the single tryptophan in angiogenin (Lee et al., 1989a). The spectrum of the PR·PRI complex differs only slightly from the sum of the spectra of each component: the overall intensities of the observed and calculated spectra are similar, although the former is slightly red-shifted.

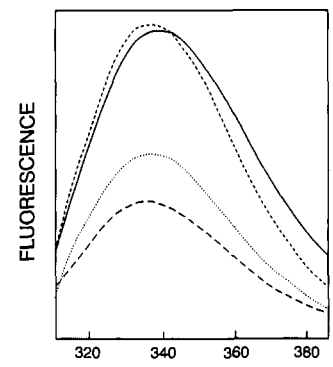

FIGURE 4: Fluorescence emission spectra of PR (--), PRI (···), and the PR·PRI complex (—) and the calculated sum of the fluorescence spectra of PR and PRI (---). Excitation was at 285 nm. Samples were 0.07 μM in 0.1 M Mes, pH 6, containing 0.1 M NaCl and 1 mM EDTA, 25 °C.

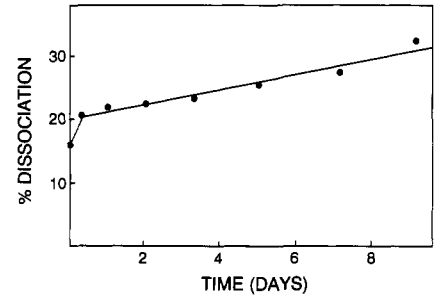

FIGURE 5: Dissociation of the PR·PRI complex. PR was incubated with 1.5 equiv of PRI for 20 min at 25 °C, followed by addition of 280 equiv of angiogenin as scavenger for free PRI. Release of free PR was then measured by assaying activity toward CpA.

(C) Association Rate. Since there is no significant fluorescence change accompanying formation of the PR·PRI complex, the apparent second-order rate constant of association,[2] k_a, was measured indirectly by examining the competition between PR and angiogenin for PRI (Lee et al., 1989a). PR and various amounts of angiogenin were mixed, PRI was added in an amount equivalent to the RNase, and the concentration of free PR was then determined by adding CpA and measuring its rate of cleavage. Both the angiogenin·PRI (Lee et al., 1989b) and PR·PRI (see below) complexes dissociate sufficiently slowly so that the relative association rates determine the partitioning of PRI between the two species. This procedure yields a rate constant of $(1.9 \pm 0.3) \times 10^8$ M^{-1} s^{-1} ($N = 7$).

(D) Dissociation Rate. The rate constant for dissociation of the PR·PRI complex was determined by first forming the complex, then adding a 280-fold excess of angiogenin to act as scavenger for free PRI, and finally measuring the amount of free PR at various times by assaying for activity toward CpA. The results are shown in Figure 5. No free PR could be measured after 9 days in a control sample lacking scavenger.

[2] Association of angiogenin and PRI follows a two-step mechanism (Lee et al., 1989a):

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

where E and I represent angiogenin and PRI, respectively. The same mechanism may pertain to the association of PRI with placental RNase. In this case, the apparent second-order rate constant of association should approximate k_2/K_i under the experimental conditions employed.

In the presence of scavenger, dissociation of 21% of the PR was detected after ~7 h, with another 12% dissociating during the subsequent 9 days. Treatment of the slower phase as a first-order process yields a dissociation rate constant of 1.8×10^{-7} s^{-1}.

Only 33% of the total PR could be detected as free enzyme by the end of the 9-day experiment. Two additional findings indicate that the remainder was still complexed with PRI and had not been inactivated irreversibly or lost due to adsorption. First, activity in a control sample lacking PRI did not change during the course of the experiment, demonstrating that free PR is stable and remains in solution. Second, full activity was recovered, measured in this case with the wheat germ RNA assay, when the PR/PRI/scavenger mixture was acidified and freeze–thawed after 9 days of incubation.

Quenching of PR Fluorescence by Acrylamide. A Stern–Volmer plot for quenching of PR fluorescence by acrylamide (not shown) is linear over the range examined (0–50 mM quencher) and yields a K_{sv} value of 62 M^{-1}, compared with 37 M^{-1} for indole. Angiogenin and PRI yield constants of 20.1 and 7.2 M^{-1}, respectively (Lee et al., 1989a). Acrylamide, 80 mM, does not influence the activity of PR toward UpA (75 μM).

Discussion

Human placental RNase inhibitor was originally isolated and characterized as an inhibitor of bovine pancreatic RNase A (Blackburn et al., 1977), although it was known that related inhibitors act on a variety of extracellular and intracellular RNases (Roth, 1967). Later, PRI was shown to inhibit human angiogenin (Shapiro & Vallee, 1987), a potent angiogenic protein that shares 33% sequence identity with RNase A, and the kinetics of the interactions of PRI with both angiogenin and RNase A were examined in detail (Lee et al., 1989a,b). These studies revealed extremely tight binding to both angiogenin and RNase A, with K_i values of 7×10^{-16} M and 4×10^{-14} M, respectively. Comparable work, however, involving RNases derived from human placenta—or indeed from any source known to contain active PRI—has not been reported. In the present study, we have therefore sought to isolate the predominant RNase species present in placenta, establish its relationship to previously identified RNases, and examine its interaction with PRI.

The superfamily of human alkaline and neutral RNases (Beintema, 1987; Beintema et al., 1988b) contains at least five distinct gene products: (i) pancreatic RNase (Weickmann et al., 1981), (ii) the "nonsecretory" RNase isolated from liver (Sorrentino et al., 1988), urine (RNase U_s; Cranston et al., 1980; Iwama et al., 1981), and eosinophils (EDN; Gleich et al., 1986), (iii) eosinophil cationic protein (ECP; Gleich et al., 1986), (iv) angiogenin (Fett et al., 1985), and (v) a uridine-specific RNase isolated from tumor cell conditioned medium and normal plasma (Shapiro et al., 1986a). All five proteins are relatively small (118–134 amino acids), acid-stable, and basic. Their amino acid sequences (Beintema et al., 1984, 1988a; Strydom et al., 1985; Kurachi et al., 1985; Rosenberg et al., 1989; Barker et al., 1989; Hamann et al., 1989; D. J. Strydom, personal communication) reveal extensive homology, with a high degree of conservation of putative active-site residues. Nonetheless, there are marked differences in reactivity and substrate specificity among the various proteins (Iwama et al., 1981; Shapiro et al., 1986a,b; Slifman et al., 1986; Gullberg et al., 1986; Sorrentino et al., 1988). For example, the activities of ECP and angiogenin toward high molecular weight RNA are ~1–2 and 5–6 orders of magnitude lower, respectively, than those of the other three RNases. In addition, the fifth RNase listed cleaves almost exclusively on the 3′ side of uridines whereas the others act at cytidines as well. Finally, some of the RNases have potent physiological activities as well, perhaps as a consequence of their particular enzymatic specificities: both EDN and ECP are neurotoxic (Durack et al., 1981; Fredens et al., 1982) and helminthotoxic (McLaren et al., 1984), and angiogenin induces neovascularization (Fett et al., 1985). Indeed, these three proteins were originally studied on the basis of their biological effects and were found to have ribonucleolytic activity only later.

The present work shows human placenta to contain a single predominant acid-stable RNase species (PR). On the basis of its structural and functional properties, this RNase appears to be identical with liver RNase, RNase U_s, and EDN.[3] Its amino acid composition and N-terminal amino acid sequence are indistinguishable from those published for preparations from the three earlier sources (Sorrentino et al., 1988; Beintema et al., 1988a; Gleich et al., 1986). Under appropriate conditions, all of these RNase isolates prefer poly(U) over poly(C) as substrate, contrasting with a strong preference of pancreatic RNase for poly(C) (Iwama et al., 1981; Sorrentino et al., 1988; R. Shapiro, unpublished results). In addition, the observed potency and specificity of PR toward dinucleotide substrates are consistent with those reported for RNase U_s (Iwama et al., 1981) and differ strikingly from those of pancreatic RNase. Thus, although PR and RNase A have similar activities toward high molecular weight RNA, the former is much less effective toward dinucleotides (Table III). For example, the k_{cat}/K_m value for cleavage of the best dinucleotide substrate, CpA, by PR is 16-fold lower than that by the pancreatic enzyme. In addition, although pancreatic RNase displays only a 10–20-fold preference for adenosine over guanosine at the N′ position, PR cleaves NpA dinucleotides ~500–1000-fold faster than the corresponding NpG. This alteration in substrate specificity may reflect the replacement of three of the pancreatic RNase residues thought to be involved in binding the N′ purine—Asn-67, Gln-69, and Glu-111 (Wodak et al., 1977; Brünger et al., 1985).[4]

As previously observed with liver RNase (Sorrentino et al., 1988), RNase U_s (Beintema et al., 1988a), and EDN (Gleich et al., 1986), PR contains amino acids at positions 7 and 17 that cannot be identified by standard sequencing procedures. The cDNA sequence of EDN (Rosenberg et al., 1989; Hamann et al., 1989) indicates that the amino acids encoded at these positions are tryptophan and asparagine, respectively. Beintema et al. (1988a) have shown that the latter residue in RNase U_s is glycosylated. However, the identity of the tryptophan derivative at position 7 has not been determined, and in fact such a posttranslational tryptophan modification appears to be without precedent. We are currently attempting to identify the modified species by the use of mass spectrometry. Meanwhile, spectroscopic information obtained in the present study may provide clues as to the nature of this derivative.

The UV absorption spectrum of PR (Figure 3) has a shape typical of tryptophan- and tyrosine-containing proteins. However, the molar absorptivity at 280 nm (19 500 M^{-1} cm^{-1}) is much greater than expected for the four tyrosines, four

[3] The placental RNase did not derive from eosinophils since histological examination revealed no eosinophils in the placental villi and only a small number of inflammatory cells in the circulatory systems.

[4] The assumption is made, both here and in the following discussion, that the polypeptide chains of PR, RNase U_s, EDN, and liver RNase are identical, as suggested above. These enzymes may differ, however, with respect to glycosylation or some other posttranslational modification.

cystines, and single unmodified tryptophan present [$\sim$12000 M^{-1} cm^{-1}, based on ϵ values for free amino acids (Beaven & Holiday, 1952)]. Since observed ϵ values for proteins generally exceed those calculated by no more than $\sim$15% (Wetlaufer, 1962), the additional absorbance presumably reflects largely the absorbance of the modified tryptophan. This derivative would thus contribute $\sim$5700–7500 M^{-1} cm^{-1} to the ϵ_{280} value of PR, i.e., an amount similar to or greater than that of tryptophan itself ($\sim$5600 M^{-1} cm^{-1}).

These observations rule out numerous potential modifications that alter the indole core structure, including ring openings, oxidations (e.g., to oxindolylalanine), and reductions (e.g., to indolinylalanine). The absorbances of oxindole and indoline between 280 and 290 nm are vastly decreased compared with that of indole (Barth et al., 1972). Addition of a hydroxyl or methoxy group at position 5, as in serotonin and melatonin, respectively, can also be excluded as possibilities, since these would result in substantially increased absorbance above 300 nm (Pappalardo et al., 1958; Barth et al., 1972). Published spectral information would also appear to rule out hydroxylations at positions 4, 6, and 7, since these modifications considerably diminish (4-OH and 7-OH) or shift (6-OH) the absorbance (Stoll et al., 1955; Julia & Pascal, 1970; Darstoor et al., 1967). Acylation of the indole nitrogen, although consistent with the UV spectrum observed (Holmquist & Vallee, 1973), can be excluded since acylated species would be degraded to tryptophan during hydrolysis in 4 N methanesulfonic acid prior to amino acid analysis. However, alkylations at various positions on the indole ring could yield stable products that have the appropriate spectral characteristics (Hinman & Lang, 1964).

Any speculations concerning the nature of the modified tryptophan in PR must also be reconciled with the observed fluorescence properties of the protein. These include (i) an emission spectrum (Figure 4) resembling that of tryptophan, with a λ_{max} at 335 nm, (ii) a relatively high quantum yield, and (iii) a K_{sv} value for fluorescence quenching by acrylamide that is even greater than that measured with free indole. The first observation is consistent with the conclusions drawn above from the UV absorbance of the enzyme, again indicating that no major alteration of the basic ring structure has occurred. Interpretation of the second observation is complicated by the presence of two distinct fluorophores and the fact that quantum yields, unlike molar absorptivities, can vary tremendously with environment. Thus, although the high quantum yield could be a direct consequence of the chemical alteration of Trp-7, it could also indicate an internal location and/or restricted mobility for the modified Trp-7, the unmodified Trp-10, or both. Concerning the latter possibilities, it should be noted that the corresponding residues of bovine pancreatic RNase—Ala-4 and Lys-7—are in relatively close proximity in three-dimensional space, with both lying in an α-helical segment encompassing amino acids 3–14 (Richards & Wyckoff, 1973). The amino acid substitutions in PR would not be expected to disrupt this structure, and in fact, Chou–Fasman calculations on EDN (Rosenberg et al., 1989) suggest that the region extending from Phe-5 to Gln-14 is α-helical. Thus, the unmodified and modified tryptophans might well be close enough to interact, thereby enhancing fluorescence substantially. Whether or not such an interaction takes place, the high quantum yield is unlikely to reflect burying of the fluorophores since Ala-4 and Lys-7 in RNase A appear to be relatively accessible to solvent (Richards & Wyckoff, 1973) and the K_{sv} value for quenching by acrylamide is extremely high.

Table IV: Kinetic Parameters of Inhibition of RNases and Angiogenin by PRI[a]

enzyme	k_a[b] (M^{-1} s^{-1})	k_d (s^{-1})	K_i (M)
PR	1.9×10^8	1.8×10^{-7}	9.4×10^{-16}
angiogenin[c]	1.8×10^8	1.3×10^{-7}	7.1×10^{-16}
RNase A[c]	3.4×10^8	1.5×10^{-5}	4.4×10^{-14}

[a] Conditions are 0.1 M Mes, pH 6.0, 0.1 M NaCl, and 1 mM EDTA, 25 °C. [b] See footnote 2. [c] From Lee et al. (1989b).

Indeed, this K_{sv} value (62 M^{-1} compared with 37 M^{-1} for indole) is perhaps the most unusual aspect of PR fluorescence. By definition, K_{sv} equals the product of k_q, the bimolecular rate constant for the quenching process, and τ_0, the fluorescence lifetime in the absence of quencher (Eftink & Ghiron, 1976, 1981).[5] In practice, K_{sv} values for tryptophans in proteins largely reflect k_q (i.e., tryptophan exposure) rather than τ_0 values, which appear to be less variable [see Eftink and Ghiron (1976) and Beecham and Brand (1985)]. Since it is inevitable that the protein at least partially obstructs collision of these tryptophans with quencher, the resultant K_{sv} values are essentially always lower than for free indole. On the basis of these considerations, a K_{sv} value for PR that is 1.7-fold higher than for indole cannot be explained simply by high exposure to solvent. Specific binding of quencher to the region around the fluorophore(s) could potentially produce such an effect. However, although such an interaction cannot be ruled out, it seems unlikely in view of acrylamide's small size, electrical neutrality, lack of effect on PR's enzymatic activity, and general tendency not to interact with proteins (Eftink & Ghiron, 1981). Alternatively, the high K_{sv} value may be a consequence of an increased value for τ_0, which could, in principle, be influenced by the chemical nature of the Trp-7 modification or by specific interactions with other residues (Beecham & Brand, 1985).

The major reason for isolating and characterizing PR was to examine its interaction with PRI. PRI and related inhibitors have been thought to play a role in the control of cytoplasmic RNA levels (Imrie & Hutchison, 1965; Kraft & Shortman, 1970). Recent findings, however, suggest an additional or alternative in vivo function involving the regulation of angiogenin, since PRI abolishes both the enzymatic and angiogenic activities of this protein (Shapiro & Vallee, 1987). Indeed, kinetic studies reveal an extraordinarily low dissociation constant of 7×10^{-16} M for the angiogenin·PRI complex, 60-fold lower than that obtained with RNase A (Lee et al., 1989a,b). In considering the relative biological importance of the interaction of PRI with angiogenin, however, a more appropriate comparison would involve human, rather than bovine, RNase and, in particular, that RNase which is present in placenta. We find that in fact PR binds to PRI with association and dissociation rate constants virtually identical with those previously measured for angiogenin, yielding a dissociation constant of 9×10^{-16} M. (The kinetic constants for the complexes of PRI with PR, angiogenin, and RNase A are compiled in Table IV.) This is a striking result given the 76% difference between the amino acid sequences of PR and angiogenin, and it suggests strong evolutionary pressure to maintain the extremely tight interactions of PRI with both proteins.

[5] It is assumed here that the slope of the Stern–Volmer plot approximates K_{sv} as defined. Additional terms related to static quenching or diffusion, which must be introduced under some circumstances, should not contribute significantly since the observed K_{sv} value is high compared with the static quenching constant for acrylamide with indole (Eftink & Ghiron, 1981) and the viscosity of the solution is low.

Dissociation of PR from its complex with PRI is biphasic (Figure 5), with a relatively rapid (~7 h) release of 21% followed by much slower dissociation of the remainder. A similar phenomenon has been observed previously, to varying extents, with the complexes of PRI with RNase A, angiogenin, and angiogenin mutants (Lee et al., 1989b; Shapiro et al., 1989; Shapiro & Vallee, 1989) and with other tight-binding inhibitors as well (Garlick & Giese, 1988). This biphasic process may reflect heterogeneity in the inhibitor, the enzyme, or both. PRI binding is sulfhydryl-dependent (Blackburn et al., 1977), and it is possible that the PRI preparation employed contained some partially oxidized protein that bound PR with decreased strength. Heterogeneity of PR with respect to glycosylation may also have contributed: there are several potential carbohydrate attachment sites (Beintema et al., 1988a), some of which may be in regions that are involved in the PRI interaction.

Blackburn and co-workers [summarized by Blackburn and Moore (1982)] have proposed that PRI binds to RNase A in the regions containing (i) Lys-7, Lys-41, Pro-42, Val-43, Lys-91, Tyr-92, and Pro-93; (ii) Lys-31 and -37; and (iii) Lys-61 and adjacent residues. Certain results obtained with angiogenin are consistent with this model; others are not. Thus, mutagenesis of the lysine corresponding to Lys-41 in RNase A decreases binding by three orders of magnitude (Lee & Vallee, 1989). In addition, the fluorescence of Trp-89 in angiogenin, which replaces Tyr-92, is greatly increased by PRI binding (Lee et al., 1989a). Several additional residues listed (e.g., Lys-31, Val-43, Lys-61, and Pro-93) are also retained or conservatively replaced in angiogenin. However, Lys-37 and -91 are substituted by serine and proline, respectively, suggesting that these RNase A residues do not participate in important interactions with the inhibitor. Involvement of the residues around Lys-61 is also questionable since (i) the primary structures of angiogenin and RNase A differ markedly in this region, (ii) an angiogenin/RNase A hybrid protein in which the angiogenin sequence 58–70 is replaced by the RNase sequence 59–73 binds PRI as tightly as does angiogenin (Harper & Vallee, 1989), and (iii) dinitrophenylation of Lys-60 in angiogenin (corresponding to Lys-61 in RNase) has no effect on the interaction with PRI (Lee & Vallee, 1989).

The extremely low dissociation constant for the PR·PRI complex—equivalent to that observed with angiogenin and 40-fold lower than that with RNase A—brings into question further aspects of this model. The primary sequence of PR differs vastly from those of both RNase A and angiogenin in the proposed PRI contact regions, with only Lys-41 conserved. The region corresponding to that around residues 91–93 of RNase A contains a two-residue insertion in PR and lacks two prolines that may be important for maintaining secondary structure in angiogenin and RNase A. Trp-89 of angiogenin, whose fluorescence is so dramatically increased upon PRI binding, is replaced in PR by a glutamine, asparagine, or isoleucine, depending on the sequence alignment chosen. Another proposed contact point, Lys-7 in RNase A (His-8 in angiogenin), is nonconservatively replaced by a tryptophan (Trp-10) in PR. Since the fluorescence quantum yield of free PR is already high, possibly due to restricted motion of Trp-10 or the tryptophan derivative at position 7 (see above), the absence of a substantial fluorescence change upon PRI binding provides no indication as to whether this region is involved in PRI binding. [Binding to PRI of either RNase A (Lee et al., 1989a) or a W89M-angiogenin mutant protein (E. A. Fox, personal communication), both of which lack tryptophan, results in a fluorescence emission spectrum similar to that of PRI alone, suggesting that the fluorescence of PRI itself is relatively unaffected by complex formation.]

The tightness of the binding of PRI to angiogenin, PR, and other members (Shapiro et al., 1986a) of the human RNase superfamily suggests an important in vivo role for the inhibitor in their regulation. In this regard, the localizations of these proteins are perhaps somewhat puzzling [see Beintema et al. (1988b)]. PRI is synthesized as a cytoplasmic protein, containing 32 free cysteine residues and no disulfides (Lee et al., 1988). All of the RNases in question, however, appear to be noncytoplasmic. Thus, they contain cystine rather than cysteine residues and are, in some instances, glycosylated. Their cDNA sequences, where known, indicate the presence of signal peptides, targeting the proteins for membrane passage. Indeed, pancreatic RNase, ECP, and EDN are components of secretory granules, and angiogenin and the uridine-specific RNase are secreted by tumor cells. [It should be noted that the use of the term "nonsecretory" to describe some of these RNases, which is prevalent in the literature (Sierakowska & Shugar, 1977), is not only inappropriate but incorrect.] In those cases where these RNases do not appear to be actively secreted, they may be lysosomal (Morita et al., 1986). The purely cytoplasmic RNases, which are, presumably, primarily responsible for degradation of mRNA and other cytoplasmic RNAs, have not been identified. Such RNases would likely be distinct from the members of the superfamily currently under consideration since, as cytoplasmic proteins, they would be expected to lack the disulfide bridges that are functionally critical for the latter. It would therefore be surprising if these RNases were effectively inhibited by PRI.

If the primary localizations of PRI and the RNases that it strongly inhibits are different, then under what circumstances do they interact in vivo? It is possible that small amounts of these RNases may leak into the cytoplasm (Beintema et al., 1988b), where the action of PRI could prevent them from degrading the various RNA species present. All of these RNases, with the exception of angiogenin and perhaps ECP, rapidly degrade mRNA, tRNA, and rRNA, and even angiogenin is very effective against 18S rRNA when it is present in intact ribosomes (St. Clair et al., 1987). On the other hand, PRI may not always be confined to the cytoplasm. Material immunoreactive toward anti-PRI antibodies is present in normal human serum; if it is PRI, then it is present at levels of 2–3 μg/mL (Feldman et al., 1988), although it appears to be largely inactive in that fluid since there is no detectable increase in RNase activity upon treatment with pHMB (F. S. Lee, personal communication). Whether its presence in serum merely reflects clearance or an active extracellular role for PRI remains to be determined. In this regard, however, it seems likely that the potent physiological effects of some RNases, including angiogenin, ECP, EDN, liver RNase (Glitz et al., 1988), and, presumably, placental RNase, might require some form of extracellular regulation.

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Peptidic Phosphonylating Agents as Irreversible Inhibitors of Serine Proteases and Models of the Tetrahedral Intermediates†

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ABSTRACT: Peptide analogues incorporating an electrophilic phosphorus moiety (**2**–**6**) have been synthesized and studied as inhibitors of a variety of serine proteases. Inhibition is irreversible and, for α-lytic protease (ALP), shown to result from covalent binding to the active site serine hydroxyl [Bone, R., Sampson, N. S., Bartlett, P. A., & Agard, D. A. (1991) *Biochemistry* (following paper in this issue)]. For reaction of human leukocyte elastase (HLE) with the thiophenyl esters **6s-V** (Boc-AAPVψ[P═O(SPh)O]AA-OMe), **4s-V** (BocAAPVψ[P═O(SPh)O]-Me), and **3s-V** (Boc-Vψ[P═O(SPh)O]AA-OMe), evidence is presented to suggest that the S_4–S_1 subsites, but not the S_1' and S_2' positions, are occupied by the inhibitors during the inactivation process. The selectivity that is observed between the proteases and the hexapeptide phosphonates **6o-V** (Boc-AAPVψ[P═O(OPh)O]AA-OMe) and **6o-F** (Boc-AAPFψ[P═O(OPh)O]AA-OMe) parallels that between these enzymes and their substrates: ALP and HLE are selectively inactivated by the Val^P-containing analogue **6o-V**, while subtilisin (SUB) shows a preference for the Phe^P derivative **6o-F**. A detailed kinetic analysis of the enzyme–inhibitor interactions was complicated by the susceptibility of the inhibitors to enzymatic degradation. The configuration at phosphorus was found not to have a significant influence on the rate at which the inhibitors react with the peptidases. Moreover, in the case of inactivation of ALP by the hexapeptide **6o-V**, the same covalent adduct is formed from both stereoisomers (Bone et al., 1991), indicating that one of these diastereomers undergoes substitution with retention of configuration.

No group of enzymes is as diverse, as ubiquitous, or as fundamental to a host of critical functions as the serine proteases (Kraut, 1977; Polgar, 1989). Divergent evolution of the chymotrypsin/trypsin family and convergent evolution of the subtilisin family are responsible for the identity of these enzymes with respect to mechanism and their diversity with respect to substrate specificity. Reflecting their varying roles as broadly or narrowly specific hydrolases, the serine proteases demonstrate sequence selectivity, through preference for certain amino acids at designated positions relative to the scissile linkage in the substrate, as well as macromolecular selectivity, through interactions over a greater range. Understanding the basis of serine protease selectivity has depended to a great extent on the structural information that has come from crystallographic studies of these enzymes and their complexes with both low and high molecular weight inhibitors (Steitz & Schulman, 1982; Barrett & Salvesen, 1986; Bode et al., 1989). Mechanistic understanding has also been advanced by these studies, since most low molecular weight inhibitors take advantage of the special reactivity of the catalytic residues in the enzyme active site (Powers & Harper, 1986; Fischer, 1988).

The classic inhibitors of the serine proteases are the phosphorylating agents, exemplified by DFP[1] (Jansen et al., 1952; Balls & Jansen, 1962; Mounter et al., 1963). These compounds react irreversibly with the active site serine hydroxyl (Ser-195 in chymotrypsin), forming a covalent adduct that has been viewed as a model for the tetrahedral intermediates involved in the normal substrate catalytic sequence (Bernhard & Orgel, 1959; Stroud et al., 1974; Kraut, 1977; Kossiakoff & Spencer, 1981). However, the traditional phosphorylating agents such as DFP bear so little resemblance to a typical peptide substrate that the utility of their enzymatic adducts as models of enzyme–substrate transition-state structures is limited. Moreover, these reagents are unable to take advantage of the substrate specificity of the different hydrolases and are therefore nonspecific. Greater selectivity is achieved by in-

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[1] Abbreviations: ALP, α-lytic protease; CHY, bovine α-chymotrypsin; HLE, human leukocyte elastase; PPE, porcine pancreatic elastase; SUB, subtilisin BPN′; DFP, diisopropyl phosphorofluoridate; DMF, dimethylformamide; DIEA, ethyldiisopropylamine; TMSiCl, trimethylsilyl chloride; TBK, triethylammonium bicarbonate; msAAPVna, methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide; sAAPAFna, succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide; bAnpe, *N*-(*tert*-butoxycarbonyl)-L-alanine *p*-nitrophenyl ester; sAPAna, succinyl-L-alanyl-L-prolyl-L-alanine *p*-nitroanilide; Tris, tris(hydroxymethyl)aminomethane; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; EDC, ethyl[(dimethylamino)ethyl]carbodiimide hydrochloride; DMAP, (dimethylamino)pyridine. Val^P and Phe^P represent the phosphonic acid analogues of valine and phenylalanine, respectively. The systematic name for Val^P would be (1-amino-2-methylpropyl)phosphonic acid and for Phe^P would be (1-amino-2-phenylethyl)phosphonic acid.

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