Degradation of Double-Stranded RNA by Human Pancreatic Ribonuclease: Crucial Role of Noncatalytic Basic Amino Acid Residues†

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ABSTRACT: Under physiological salt conditions double-stranded (ds) RNA is resistant to the action of most mammalian extracellular ribonucleases (RNases). However, some pancreatic-type RNases are able to degrade dsRNA under conditions in which the activity of bovine RNase A, the prototype of the RNase superfamily, is essentially undetectable. Human pancreatic ribonuclease (HP-RNase) is the most powerful enzyme to degrade dsRNA within the tetrapod RNase superfamily, being 500-fold more active than the orthologous bovine enzyme on this substrate. HP-RNase has basic amino acids at positions where RNase A shows instead neutral residues. We found by modeling that some of these basic charges are located on the periphery of the substrate binding site. To verify the role of these residues in the cleavage of dsRNA, we prepared four variants of HP-RNase: R4A, G38D, K102A, and the triple mutant R4A/G38D/K102A. The overall structure and active site conformation of the variants were not significantly affected by the amino acid substitutions, as deduced from CD spectra and activity on single-stranded RNA substrates. The kinetic parameters of the mutants with double-helical poly(A)·poly(U) as a substrate were determined, as well as their helix-destabilizing action on a synthetic DNA substrate. The results obtained indicate that the potent activity of HP-RNase on dsRNA is related to the presence of noncatalytic basic residues which cooperatively contribute to the binding and destabilization of the double-helical RNA molecule. These data and the wide distribution of the enzyme in different organs and body fluids suggest that HP-RNase has evolved to perform both digestive and nondigestive physiological functions.

Extracellular ribonucleases (RNases)[1] from tetrapods constitute a large superfamily of rapidly evolving proteins (*1*, *2*). The prototype of this superfamily is bovine pancreatic RNase A (see, for a recent review, ref *3*); its function in ruminant and ruminant-like species is to degrade dietary RNA (*4*, *5*). However, many other members of the superfamily evolved to perform possible extracellular nondigestive functions (*6*). Several of these proteins possess special, noncatalytic biological actions dependent upon their ribonucleolytic activity, such as neurotoxicity, angiogenic activity, immunosuppressivity, and antiviral and antitumor action (*7*).

Mammalian ribonucleases, on the basis of their structural and functional properties, have been grouped into four different RNase families (*2*, *8*−*10*). According to this classification, the two main RNase groups, the so-called "secretory" and "nonsecretory" ribonucleases (*11*), have been more properly designated (*8*, *10*) pancreatic-type (pt) and nonpancreatic-type (npt) RNases, respectively. All ptRNases found in mammalian pancreas, as well as in other organs, are products of orthologous genes, have a pH optimum on RNA around 8.0, prefer poly(C) over poly(U) as a substrate, and hydrolyze 2′,3′-cyclic nucleotides; nptRNases (only found in tissues other than pancreas and structurally 30−35% identical to ptRNases) show a pH optimum on RNA around 6.5−7.0, prefer poly(U) over poly(C), and do not hydrolyze 2′,3′-cyclic nucleotides (*2*, *10*). The ribonucleases which belong to a group of evolutionarily highly conserved proteins, named RNases 4 (*12*), constitute the third RNase family; these enzymes, sharing some catalytic properties with both pt- and nptRNases, have been also referred to as pt/nptRNases (*8*, *10*). All mammalian RNases structurally and functionally similar to human angiogenin (*13*, *14*) have been grouped into the fourth RNase family.

To date, eight structurally distinct ribonucleases have been identified in humans: RNase 1 from pancreas and other organs, or HP-RNase (*15*, *16*); RNase 2 (*17*), also named EDN (*18*), identical to liver/spleen RNase (*19*, *20*); RNase 3, named ECP (*21*), only expressed in eosinophils (*22*); RNase 4 from colon carcinoma and normal plasma (*23*, *24*); RNase 5, or angiogenin (*13*); RNase 6 or RNase K6 (*25*); RNase 7, a novel epithelial-derived antimicrobial protein (*26*), also expressed in various somatic tissues (*27*); and the recently discovered RNase 8, expressed uniquely in placenta (*28*).

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[1] Abbreviations: RNase(s), ribonuclease(s); EDN, eosinophil-derived neurotoxin; ECP, eosinophil cationic protein; poly(A), poly(adenylic acid); poly(C), poly(cytidylic acid); poly(A)·poly(U), polyadenylic-polyuridylic acid (double-stranded homopolymer); poly(dA-dT)·poly(dA-dT), polydeoxyadenylic-thymidylic acid (double-stranded alternating copolymer); Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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In humans all ptRNases found in various organs, at similar or higher levels than in pancreas (*29*), are products of the same RNase 1 gene (*30*); all nptRNases (i.e., RNases 2, 3, 6, 7, and 8) are products of closely related genes. However, the possible nondigestive functions performed in body fluids and tissues other than pancreas by RNases are poorly understood. In this regard, it is worth mentioning that HP-RNase constitutes a significant proportion (70−80%) of all RNases contained in human serum, urine, milk, saliva, and seminal plasma (*29*, *31*−*33*). Moreover, it has been found recently (*34*) that human endothelial cells selectively express large amounts of RNase 1, a finding that can provide a possible answer to the controversial origin of HP-RNase in blood serum and other fluids. It was also suggested that a significant part of the enzyme present in various organs could originate from the endothelium (*34*).

HP-RNase shows a great catalytic versatility, and thanks to its ability to degrade under physiological salt conditions both single- and double-stranded RNA as well as poly(A) (*8*, *35*, *36*), it may have important extracellular functions. HP-RNase is the most powerful enzyme to degrade dsRNA within the tetrapod RNase superfamily (*35*). Therefore, an understanding of the structural determinants of this peculiar activity may be an important step toward the comprehension of the possible biological functions of HP-RNase. According to a mechanism proposed by Libonati and Sorrentino (*8*, *36*, *37*), the degradation of dsRNA by HP-RNase (and other mammalian ptRNases) is based on a local destabilization of the nucleic acid secondary structure induced by the enzyme protein. This was in turn related to the number and location of positive charges displayed by the RNase molecule in its interaction with the polyanionic double-helical substrate (*35*). To verify this hypothesis, we prepared by site-directed mutagenesis four HP-RNase variants and measured, in side-by-side experiments, their activities on both single- and double-stranded polyribonucleotides, as well as their helix-destabilizing action on a synthetic DNA substrate. The results reported here allow to relate the peculiar surface positive electrostatic potential of HP-RNase to its ability to destabilize and degrade double-helical RNA molecules. A preliminary report of this study was presented at the Sixth International Meeting on Ribonucleases (*38*).

EXPERIMENTAL PROCEDURES

Materials. Poly(A), poly(C), poly(U), yeast RNA, poly(A)·poly(U), and bovine RNase A (type XIIA) were all Sigma products. Poly(dA-dT)·poly(dA-dT) was purchased from Amersham Pharmacia Biotech. High molecular weight double-stranded RNA from f2 *sus 11* virus, prepared as described (*39*), was a gift of Dr. J. Doskocil (Czechoslovak Academy of Sciences, Praha). Single-stranded viral RNA (ssRNA) was obtained by thermal denaturation of f2 *sus 11* dsRNA in 1 mM EDTA, pH 8.0 (*36*). The other chemicals were of analytical grade. Assay buffer solutions were freshly prepared using distilled water, treated with a MilliQ water purification system, under sterile/RNase-free conditions, and always filtered before use.

Molecular Modeling. The structural model for HP-RNase was prepared with the aid of SWISS-MODEL (*40*), a freely accessible server for automated comparative protein modeling (http://www.expasy.ch/swissmod/SWISS-MODEL.html), and was based on the atomic coordinates of the crystal structures of two HP-RNase variants (Protein Data Bank accession codes 1DZA and 1E21). The model for HP-RNase in complex with the tetranucleotide d(pApTpApA) was obtained with the program SWISS-Pdb Viewer (*41*) by superimposing the HP-RNase model and the crystal structure of RNase A in complex with the tetranucleotide (*42*).

Preparation of Mutant Proteins. Site-directed mutagenesis of the synthetic DNA that codes for HP-RNase (*43*) was performed by the overlap extension method of Ho et al. (*44*). Mutant DNAs were sequenced to establish the presence of the programed mutations and rule out any spurious changes. Mutant proteins were expressed in *Escherichia coli*, extracted from the inclusion bodies, refolded, purified, and structurally characterized as previously described (*45*).

Circular Dichroism Measurements. Circular dichroism (CD) spectra of all purified recombinant proteins were recorded at 25 °C using a JASCO 715 spectropolarimeter equipped with a thermostatically controlled cuvette holder (JASCO PTC-348). All measurements were carried out in 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, by using a quartz cell with a path length of 0.1 cm. The raw CD measurements (in millidegrees) were converted to molar ellipticity $[\Theta]$ and mean residue ellipticity $[\Theta]_m$ (in deg cm^2 dmol^{-1}) using molecular weight values per residue.

Assays of RNase Activity. Ribonucleolytic activity against yeast RNA [purified from the commercial product according to Blackburn et al. (*46*)] was measured at 25 °C and pH 5.0 by the spectrophotometric assay of Kunitz (*47*) with 0.6 mg/mL RNA. The cleavage of single- and double-stranded synthetic polyribonucleotides as well as viral ssRNA and dsRNA was monitored by UV spectroscopy as described (*35*, *36*). Substrate concentrations (in terms of phosphodiester groups) were determined by ultraviolet absorption using the following extinction coefficients at pH 7.5: $\epsilon_{257} = 10000$ M^{-1} cm^{-1} for poly(A), $\epsilon_{268} = 6200$ M^{-1} cm^{-1} for poly(C), and $\epsilon_{261} = 9430$ M^{-1} cm^{-1} for poly(U) (*48*); $\epsilon_{260} = 6500$ M^{-1} cm^{-1} for poly(A)·poly(U) (*35*). Briefly, the substrate and the appropriate amount of enzyme were mixed in 1 mL of 0.1 M Mops, pH 7.5, containing 0.1 M NaCl, at 25 °C. Absorbance increases associated with substrate cleavage were continuously monitored at 260 nm as a function of time, with a Varian Cary 1E spectrophotometer, equipped with a temperature controller. Absorbance changes per minute (deduced from the slope of the linear part of the recordings) were converted to initial reaction velocities by using the following $\Delta\epsilon$ values, calculated from the difference in millimolar absorptivity of each synthetic polymeric substrate at 260 nm and those of the degradation products measured after 6 and 12 h at 25 °C, i.e., at the end of the RNase-catalyzed transphosphorylation reaction: $\Delta\epsilon_{260} = 3.45$ mM^{-1} cm^{-1} for poly(A); $\Delta\epsilon_{260} = 1.25$ mM^{-1} cm^{-1} for poly(C); $\Delta\epsilon_{260} = 0.65$ mM^{-1} cm^{-1} for poly(U); $\Delta\epsilon_{260} = 3.4$ mM^{-1} cm^{-1} for poly(A)·poly(U). All assays were performed in duplicate with two different enzyme preparations. The specific activities of the RNases tested with poly(A), poly(C), and poly(U) as substrates were expressed as units per milligram of protein. One enzyme unit was defined as the amount of RNase that catalyzes the cleavage of 1 μmol of phosphodiester linkages of the single-helical substrate per minute at 25 °C. With viral ssRNA and dsRNA as substrates,

Table 1: Correlation between Location of Basic and Acidic Residues in Mammalian ptRNases and Activity on dsRNA[a]

	position							relative activity on	
ribonuclease	4	6	32	34	38	39	102	dsRNA	yeast RNA
bovine pancreas	Ala	Ala	Ser	Asn	Asp*	**Arg**	Ala	1	1
giraffe pancreas	Ala	Ala	Ala	Asn	Asp*	**Arg**	Ala	1	1
human pancreas	**Arg**	**Lys**	**Arg**	Asn	Gly	**Arg**	**Lys**	300	0.3
whale pancreas	Pro	Met	**Arg**	**Lys**	Gly	**Arg**	**Lys**	32	0.3
pig pancreas	Pro	**Lys**	**Arg**	Asn	Gly	**Arg**	Glu*	24	0.5
bovine brain	Ala	Ala	**Arg**	**Arg**	Gly	**Arg**	**Lys**	22	0.8
r-giraffe brain[b]	Ala	Ala	**Arg**	**Arg**	Gly	**Arg**	**Lys**	21	0.7

[a] Modified from Libonati and Sorrentino (*35*). Basic amino acid residues are indicated in boldface type. Acidic residues are marked with asterisks. [b] Recombinant giraffe brain RNase was a gift of Drs. E. Confalone and A. Furia.

units were defined as absorbance change per minute divided by total measurable change at 260 nm (*36*).

Steady-State Kinetic Analyses. The kinetic parameters for the degradation of poly(A)·poly(U) were determined by Lineweaver−Burk plots using the initial velocity data obtained under the assay conditions described above. Substrate concentrations were from 40 to 240 μM; enzyme concentrations were 11.6 nM (0.17 μg/mL) for all HP-RNases and 2.5 μM (34 μg/mL) for RNase A.

Thermal Denaturation of Nucleic Acids. Thermal transition profiles of poly(dA-dT)·poly(dA-dT) were determined in the presence and absence of RNases. Measurements were made at 260 nm in thermostatically controlled stoppered cuvettes with a Varian Cary 1E spectrophotometer in a temperature range of 30−85 °C. Experiments were performed in 10 mM Mops, pH 7.5, containing 0.1 M NaCl or 0.05 M NaCl, with a polynucleotide concentration of about 11 μg/mL (initial A_{260nm} = 0.230); when present, RNase was about 11 μg/mL, and its contribution to the absorbance of the mixture at 260 nm was negligible or nil.

RESULTS AND DISCUSSION

Design of HP-RNase Variants. The aim of this work was to verify the relationship between content of specific basic residues in a ribonuclease and its ability to degrade dsRNA (*8*, *36*, *37*, *49*). HP-RNase was chosen as a model for its potent activity on this substrate which may have important physiological implications. The project of HP-RNase mutants was guided by a comparative analysis of the amino acid sequence of the human enzyme (see Table 1) with those of mammalian ptRNases less active on dsRNA to detect possible correlations between location of basic or acidic residues and activity against dsRNA (*35*). In fact, HP-RNase, whose activity on dsRNA is 2 orders of magnitude higher than that of bovine RNase A (Table 1), has four basic amino acids (Arg-4, Lys-6, Arg-32, Lys-102) at positions where RNase A has instead neutral residues. At the same positions, the other RNases, which are about 20−30 times more active than RNase A on dsRNA (Table 1), possess two or three variously located basic residues. Interestingly, number and location of positive charges relate to degrading activity on dsRNA but not to that on yeast RNA. Furthermore, while bovine and giraffe pancreatic RNases (virtually inactive on dsRNA) have an aspartate residue at position 38, close to the positive side chain of Arg-39, HP-RNase and the other ptRNases active on dsRNA possess at the same position a glycine residue (*35*). Hence, besides basic residues, also the presence of a negative charge or a glycine at position 38,

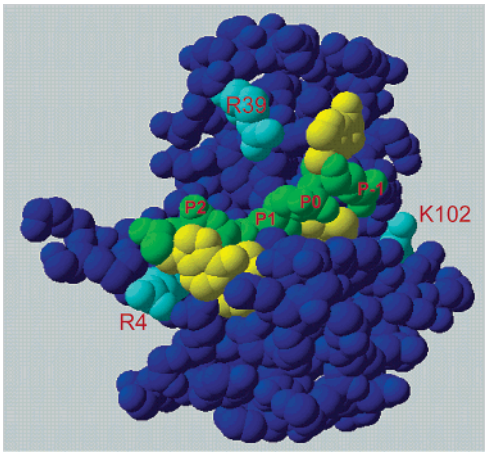

FIGURE 1: Molecular model for HP-RNase in complex with the tetranucleotide d(pApTpApA). Each atom is represented by its van der Waals surface. The oligonucleotide is shown in green (sugar−phosphate backbone) and yellow (bases); basic residues are shown in turquoise.

with a likely effect on the Arg-39 side chain, may play a role. It must be noticed that Arg-39 is one of the nine basic amino acids that in RNase A (and most likely in all ptRNases) were believed to form an extended multisite cationic region involved in protein−nucleic acid interactions (*50*).

These observations led to the hypothesis that in a ribonuclease "active on dsRNA" specifically located basic residues, together with the lack of a negative charge and/or the presence of a glycine at position 38, could contribute to make the enzymatic degradation of the polyanionic double-helical substrate more efficient (*35*). In particular, in HP-RNase (see Figure 1), it was found by modeling that two of these basic residues (Arg-4 and Lys-102), but also Arg-39, are located on the periphery of the main phosphate binding subsites, which would increase the local electrostatic potential of that region.

On the basis of the considerations made above, to verify the possible role of noncatalytic basic residues in the mechanism of dsRNA degradation, we selected three target residues in HP-RNase: Arg-4, Gly-38, and Lys-102. We replaced these residues in the sequence of the human enzyme with the corresponding amino acids of the bovine RNase A sequence (Ala-4, Asp-38, and Ala-102) to yield three single HP-RNase mutants: R4**A**, G38**D**, and K102**A**. We also designed a fourth HP-RNase variant by replacing all three target residues to form the triple mutant, R4**A**/G38**D**/K102**A**, henceforth termed **ADA**.

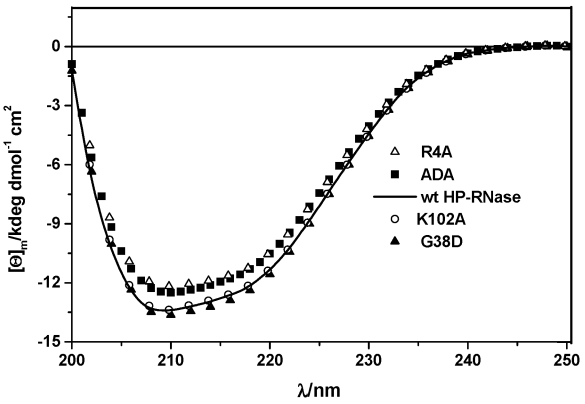

FIGURE 2: Far-UV CD spectral analysis of wild-type HP-RNase (solid line) and its four variants at 25 °C. Spectra are presented as mean residue ellipticity [Θ], expressed in deg cm^2 $dmol^{-1}$.

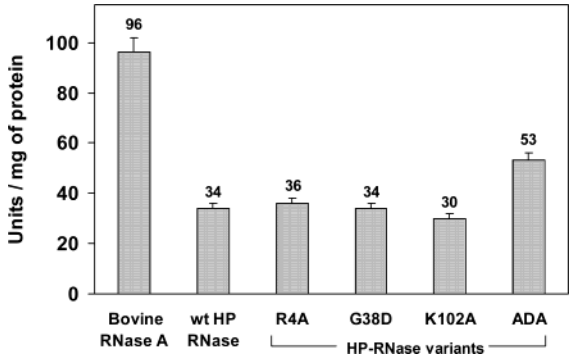

FIGURE 3: Activity of RNase A, wt HP-RNase, and HP-RNase variants toward yeast RNA. Kunitz assays were performed in duplicate at 25 °C, pH 5.0. Each value is the mean of three individual experiments.

Expression, Purification, and Structural Characterization of HP-RNase Variants. Mutant cDNAs coding for the three single HP-RNase variants (R4A, G38D, K102A) and the triple mutant ADA were prepared by PCR and expressed in *E. coli*; the recombinant proteins were then recovered from inclusion bodies, refolded, purified, and structurally characterized as previously described (*45*). In addition, the purified proteins were analyzed by circular dichroism (CD). As shown in Figure 2, all four HP-RNase mutants appear to be folded in a typical $\alpha + \beta$ conformation, with CD spectra very similar to that of the recombinant wild-type protein. In particular, no alteration was found in the spectrum of the mutant G38D despite the lack of a glycine residue at position 38, which in the recently determined three-dimensional structure of a HP-RNase variant (*51*) was found to be responsible for a greater conformation flexibility at the loop $\alpha 2\beta 1$ in comparison with the same region of bovine RNase A. The melting temperatures of all the mutants were found to be around 58 °C, very similar to that of the wild-type protein (data not shown). These results indicated that the overall structure of the four HP-RNase variants was not significantly affected by amino acid replacements.

Catalytic Characterization of HP-RNase Variants: Action on Yeast RNA and Single-Stranded Polyribonucleotides. The ribonucleolytic activities of wt HP-RNase and its four mutants toward yeast RNA, poly(C), and poly(U) were tested in side-by-side spectrophotometric assays. Bovine RNase A was included for reference. The results summarized in Figures 3 and 4 indicate that the selected mutations did not greatly alter the efficiency of the transesterification reaction catalyzed by the human enzyme toward single-stranded RNA substrates. In fact (see Figure 3), the three single HP-RNase mutants (R4A, G38D, K102A) as well as the triple mutant ADA, in the standard Kunitz assay with yeast RNA as a substrate, were shown to have activity values very similar to that of wt HP-RNase. It is worth noticing here that the recombinant wt HP-RNase (Figure 3), in agreement with previously reported results (*36*) obtained with the enzyme purified from human pancreas, seminal plasma, or urine, degraded yeast RNA at one-third the rate of bovine RNase A.

Qualitatively similar results were obtained with poly(C) and poly(U) as substrates (see Figure 4). The amino acid replacements had only little or no effect on the ribonucleolytic activity of HP-RNase against these single-stranded homopolymers. In fact, the three single mutants (R4A, G38D, K102A) displayed on both substrates a similar or higher activity in comparison with that of wt HP-RNase, whereas the triple mutant ADA showed 62% or 83% activity on poly(C) or poly(U), respectively, as compared to that of the wild-type enzyme. However, the modest reduction of specific activity, shown by the triple mutant ADA on these polyribonucleotides, did not affect the substrate preference of the enzyme for poly(C) over poly(U), typical of HP-RNase and RNase A. It should be noted that the residues of the B1 subsite (Thr-45 and Asp-83) are all conserved between the bovine and human enzymes (*3*, *15*, *16*, *52*).

Activity of HP-RNase and Its Mutants against Poly(A). Homopolymeric substrates such as poly(C) and poly(U) are efficiently degraded by all mammalian ptRNases, presumably because of their effective binding to all available subsites (B1, B2, B3) of the enzyme molecule. In particular, the B1 subsite binds only pyrimidine bases which, although with lower affinity, can be accepted also by the B2 and B3 subsites (reviewed in ref *3*). Poly(adenylic acid), instead, is a poor substrate for both RNase A and HP-RNase because of the presence of Thr-45 in the B1 subsite that sterically excludes purine bases which can be accepted only by B2 and B3 subsites (*3*, *52*). Thus, to measure the activity of HP-RNase on poly(A), a large excess of enzyme [100-fold higher than that for poly(U)] had to be used (*36*).

When recombinant wt HP-RNase and the four variants were tested against poly(A) as a substrate, the three single HP-RNase variants were found to display activity values very similar to that of the wild-type enzyme. The replacement, instead, of all three target residues of HP-RNase with the corresponding amino acids of RNase A in the triple mutant ADA reduced the specific activity of the enzyme toward poly(A) by a factor of 5.6. These results support the hypothesis (*36*) that relates the higher activity of HP-RNase (relative to RNase A) on polyadenylic acid with the presence of additional positive charges on the periphery of the main phosphate binding subsites of the human enzyme, which are all conserved between HP-RNase and RNase A. On the other hand, the slight difference found in the position of Lys-66 (P0) within the three-dimensional structure of a HP-RNase variant, in comparison with that of RNase A (*51*), is not sufficient to explain this enhanced activity.

The relationship between local positive electrostatic potential and high activity on poly(A) is also supported by the observation that native HP-RNase purified from urine, which

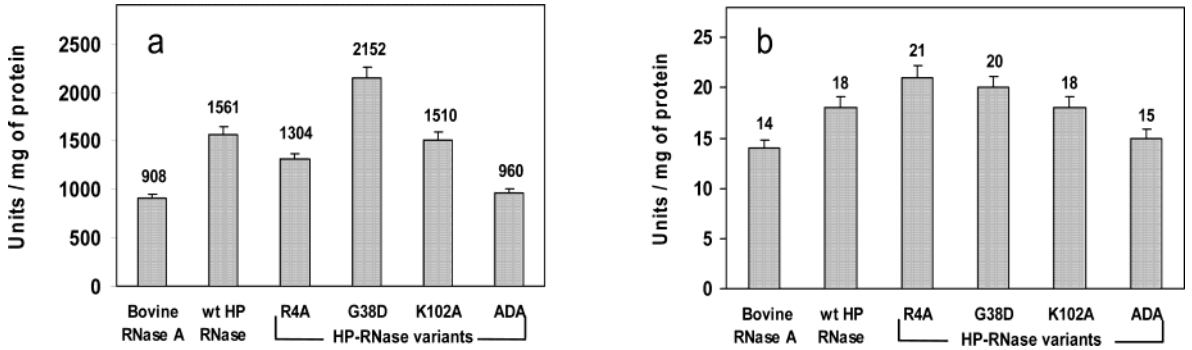

FIGURE 4: Activity of RNase A, wt HP-RNase, and HP-RNase variants toward poly(C) (panel a) and poly(U) (panel b). Spectrophotometric assays were performed in duplicate at 25 °C, pH 7.5. The values are presented as the means of three individual experiments.

contains negatively charged sugars, was found to be about 15-fold less active on poly(A) than the enzyme isolated from pancreas or seminal plasma (*36*).

Activity of HP-RNase and Its Variants against Synthetic and Natural Double-Stranded Polyribonucleotides. RNase A and the other RNases of the superfamily split phosphodiester bonds in RNA by a transesterification mechanism which is dependent upon a linear arrangement of the phosphorus atom, the 2′-oxygen acting as an internal nucleophile and the 5′-oxygen leaving group (*3*, *53*). The structure of dsRNA, in its canonical A-form, does not meet the stereochemical requirements for the "in line" mechanism of the enzymatic degradation of RNA; this explains why this RNA species is in general resistant to RNase action (*35*).

The ability of some mammalian ptRNases to cleave dsRNA at physiological ionic strength has been related to the considerable nucleic acid helix-destabilizing activity shown by these proteins (*8*, *35*−*37*). Furthermore, mammalian ptRNases, such as HP-RNase, endowed with a surface positive electrostatic potential stronger than that of bovine RNase A, were shown to display helix-unwinding action under conditions in which RNase A had no such activity (*35*, *36*). Thus, the idea was advanced that the ability of a ribonuclease to degrade dsRNA could be dependent on the specific density of basic amino acids on the surface of the protein, which would improve the helix-destabilizing efficiency of the enzyme (*10*, *35*). This would engender a preferential binding to short single-stranded sequences of the dsRNA substrate transiently exposed by spontaneous thermal fluctuations (*54*, *55*).

The analysis of the structural determinants of the activity of HP-RNase on dsRNA was carried out as follows. We determined the steady-state kinetic parameters for the degradation of poly(A)·poly(U) by the action of the four HP-RNase variants in comparison with those of the wild-type enzyme and bovine RNase A. The results summarized in Figure 6 show that the substitution of a single residue in the HP-RNase variants R4A, G38D, and K102A reduced the specificity constant of the enzyme, k_{cat}/K_m, for the double-helical substrate by a factor of about 2.5. This decrease, as it appears in Table 2, is mainly due to a 2−3-fold increase of the K_m value. Hence, the three target residues, individually, do not significantly contribute to the k_{cat}/K_m value of HP-RNase (Figure 6) in comparison with that of RNase A. In fact, the recombinant wild-type human enzyme (Table 2), in agreement with the results obtained with native HP-RNase (*36*), was found to be about 500-fold more active than bovine

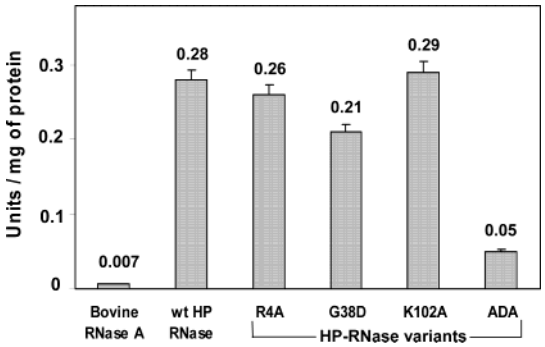

FIGURE 5: Activity of RNase A, wt HP-RNase, and HP-RNase variants toward poly(A). Spectrophotometric assays were performed in duplicate at 25 °C, pH 7.5. Enzyme concentrations were 7−20 μg/mL for wt HP-RNase and its variants and 116 μg/mL for RNase A. Each value is the mean of two individual experiments.

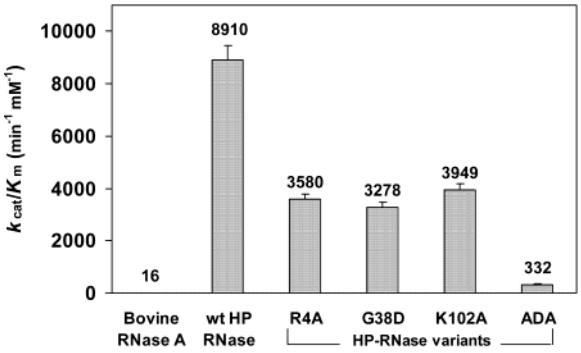

FIGURE 6: Specificity constants of RNase A, wt HP-RNase, and HP-RNase variants for the cleavage of poly(A)·poly(U) determined at 25 °C, pH 7.5. Enzyme concentrations were 0.17 μg/mL for wt HP-RNase and its variants and 34 μg/mL for RNase A. The values are presented as the means of three individual experiments.

RNase A on this substrate. However, the considerable difference of activity between bovine RNase A and HP-RNase for the cleavage of poly(A)·poly(U), at physiological salt conditions, is essentially determined by their largely different k_{cat} values. It should be noted that, while the K_m value of HP-RNase (Table 2) is only 3.7-fold lower than that of RNase A, the k_{cat} value of HP-RNase is about 150-fold higher than that of the bovine enzyme. For a ribonuclease, the k_{cat} value depends on the conformation of the cleavable phosphodiester bond of the RNA substrate bound at the RNase active site (*56*). Moreover, as expected from the multiple Coulombic interactions between a cationic enzyme and a polyanionic substrate molecule, the k_{cat} value is highly influenced by salt concentration. In fact, a previously published study (*57*) of the dependence of kinetic

Table 2: Kinetic Parameters for the Cleavage of Poly(A)·Poly(U) by Bovine RNase A, wt HP-RNase, and HP-RNase Variants

ribonuclease	K_m[a] (mM)	k_{cat}[a] (min^{-1})	k_{cat}/K_m relative to RNase A
RNase A	0.376 ± 0.032	6 ± 0.53	1
wt HP	0.100 ± 0.011	891 ± 40	557
R4A	0.314 ± 0.035	1124 ± 139	224
G38D	0.209 ± 0.021	685 ± 86	205
K102A	0.389 ± 0.028	1536 ± 75	247
ADA	0.244 ± 0.027	81 ± 12	21

[a] Values determined by Lineweaver−Burk plots using initial velocity values obtained in duplicate or triplicate with 10 different substrate concentrations. Enzyme concentrations were 11.6 nM (0.17 μg/mL) for all HP-RNases and 2.5 μM (34 μg/mL) for RNase A. Correlation coefficients were higher than 0.99. Data represent the means (±SE) of three individual experiments.

parameters on ionic strength for the degradation of poly(A)·poly(U) and poly(U) by RNase A showed that, at physiological salt concentration, the k_{cat} value for poly(A)·poly(U) dramatically decreased while that for the single-stranded substrate poly(U) reached its maximum. In addition, no significant differences were observed in the influence of ionic strength on the K_m values measured with poly(A)·poly(U) as compared to those determined with poly(U).

Thus, on the basis of the above considerations, it can be stated that none of the three selected residues plays a crucial role in determining, under physiological salt conditions, the potent activity of HP-RNase against dsRNA. On the contrary, it has been recently reported that glycine-38 is the main determinant of the ribonucleolytic activity of human pancreatic ribonuclease on dsRNA (*58*). The authors found that the mutation of Gly-38 to alanine or aspartic acid resulted in a 3-fold reduction of the dsRNA degrading activity of HP-RNase. Although their data are in line with ours (G38D was found to be 2.7-fold less active), we would not agree with their conclusion that glycine-38 is extremely crucial for the cleaving activity of human pancreatic RNase against dsRNA (*58*). We believe that in HP-RNase (as well as in other mammalian ptRNases active on dsRNA) Gly-38 has an important but not crucial function. Possibly, it may enhance the role of Arg-39 as one of the many noncatalytic phosphate binding residues involved in the interaction of the RNase protein with the double-helical substrate. Hence, the importance of Gly-38 in HP-RNase seems to be based on both the lack of a negative charge at position 38 and the greater conformational flexibility at the loop α2β1 of the human enzyme structure (*51*). This would partially improve the helix-unwinding ability of the enzyme.

These observations are in agreement with the results obtained with a monomeric RNase A mutant (*59*) with glycine at position 38, which was found to be only 5 times more active than the native enzyme toward dsRNA. In addition, the high activity on dsRNA of a dimeric RNase A variant (*60*) bearing the mutation D38G was interpreted as the result of the combined effects of both glycine-38 and dimeric structure, which would generate a higher positive charge density in discrete regions of the enzyme molecule. According to the model presented here, the increase in dsRNA degrading activity shown by the dimeric RNase A variant, as already proposed (*35*, *60*), would be related to the higher local electrostatic potential of the enzyme more than to the presence of two active sites which bind to a RNA strand at the same time, as proposed by other authors to explain the activity of bovine seminal RNase on dsRNA (*59*).

Table 3: Activity of wt HP-RNase, Its Triple Mutant ADA, and Bovine RNase A on Viral Single- and Double-Stranded RNA

ribonuclease	activity on ssRNA (units/mg)[a]	activity on dsRNA (units/mg)[a]	relative activity[b]
wt HP	18809 ± 673	113 ± 13	100
ADA	16025 ± 1269	8.2 ± 0.85	7.2
RNase A	12350 ± 1626	0.97 ± 0.16	0.85

[a] Each value is the mean (±SD) of measurements performed at 25 °C in 0.1 M Mops, pH 7.5, containing 0.1 M NaCl, with 40 μg/mL of RNA. Enzyme concentrations were 8−10 ng/mL with ssRNA as a substrate and 1.4 (wt HP-RNase), 10 (ADA), and 86 μg/mL (RNase A) with dsRNA as a substrate. Units were defined as absorbance change per minute divided by total measurable change (*36*). [b] Percent of wt HP-RNase activity on dsRNA.

The results obtained with the triple HP-RNase variant ADA shown in Figure 6 strongly support our hypothesis based on the electrostatic potential of the enzyme. The replacement of all three residues resulted indeed in a 27-fold reduction of the specificity constant of HP-RNase for the degradation of the double-helical substrate, which suggests that the effect is more than additive. Moreover, the decrease in k_{cat}/K_m of the triple mutant (Figure 6) is essentially ascribable to the drastic diminution of the k_{cat} value (see Table 2) since the K_m of the mutant ADA was found to be very similar to those of the three single mutants and only 2.5-fold higher than that of wt HP-RNase. The finding that the triple HP-RNase variant ADA is 21-fold more active than RNase A can be justified by the presence in the human enzyme of other basic amino acids (for example, Lys-6 and Arg-32; see Table 1) at positions where RNase A has neutral residues. This conclusion is strengthened by the observation that pig pancreatic RNase (Table 1) has basic residues at positions 6 and 32 and displays on dsRNA a 24-fold higher activity than RNase A (*35*). The observation that the activity of HP-RNase against poly(U) was not significantly affected by the triple amino acid substitution (Figure 4b), while the activity of ADA on poly(A)·poly(U) was 27-fold reduced (Figure 6), indicates that the phenomenon is to be essentially attributed to the double-helical nature of the substrate. As for the 5.6-fold lower activity of ADA on poly(A), it should be noted that in this assay the enzyme concentration was at least 100-fold higher than that used in the RNase assays against both poly(U) and the double-stranded substrate. In the assay on poly(A)·poly(U) indeed, the concentration of the human RNases was 0.17 μg/mL, and at this enzyme concentration, no degradation of poly(A) by ADA could be detected (data not shown). Thus, the lower activity of ADA against poly(A)·poly(U) was essentially ascribable to its reduced binding and degrading efficiency of the poly(U) strand within the secondary structure of the synthetic dsRNA-like substrate molecule. In conclusion, the data discussed above indicate that the activity of HP-RNase on poly(A)·poly(U) is related to the presence of basic residues which cooperatively contribute to the binding efficiency of the enzyme to the double-helical substrate.

These conclusions were strengthened by the results of experiments carried out on a natural, high molecular weight dsRNA substrate. The data tabulated in Table 3 show that the activity of recombinant wt HP-RNase is 2 orders of

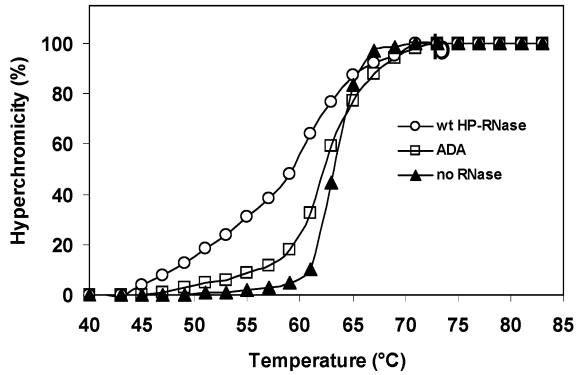

FIGURE 7: Effect of wt HP-RNase and its triple mutant ADA on the thermal transition profile of poly(dA-dT)·poly(dA-dT). Double-stranded polymer alone (▲) or nucleic acid−protein complexes were spectrophotometrically analyzed as a function of temperature (from 30 to 85 °C) in 10 mM Mops, pH 7.5, containing 0.05 M NaCl.

magnitude higher than that of RNase A on this substrate, as found with poly(A)·poly(U) as a substrate. Likewise, just as with poly(A)·poly(U), the triple mutant ADA has a much reduced degrading activity, about 7% of the activity of wt HP-RNase. On ssRNA, obtained by thermal denaturation of the viral f2 *sus 11* dsRNA, instead, the activity of the wild-type human enzyme and its triple mutant ADA were found to be very similar, in agreement with the results obtained with synthetic single-stranded polymeric substrates.

Helix-Destabilizing Activity of HP-RNase and Its Variants against a Synthetic DNA Substrate. The helix-destabilizing ability of HP-RNase is well-known and has been analyzed in comparison with that of RNase A and other RNases under different pH and ionic strength conditions. In these assays, a good correlation was usually found between the activity of a ribonuclease on dsRNA and its ability to bind and destabilize duplex DNA (*36*, *61*, *62*).

To verify the proposed relationship between the helix-unwinding activity of HP-RNase and the presence of basic residues in discrete regions of the RNase molecule (*35*), we tested the effect of wt HP-RNase and its mutants on the thermal melting of poly(dA-dT)·poly(dA-dT). The data were obtained in experiments carried out at pH 7.5 under different ionic strength conditions: in 0.1 M NaCl (data not shown) and 0.05 M NaCl (see Figure 7). We found that the higher the destabilizing effect (that of wt HP-RNase), the higher the activity on dsRNA; the lower the destabilizing effects (those of HP-RNase variants), the lower the activity. In Figure 7, the thermal transition profile of the double-stranded polymer is compared with those obtained in the presence of ribonucleases, under conditions in which (*59*, *61*, *62*) the DNA binding and the relative helix-destabilizing effects of wt HP-RNase and its triple mutant ADA could be more clearly observed. From the data illustrated in Figures 6 and 7 it is evident that both helix-destabilizing ability and activity on dsRNA relate to the presence of basic amino acids on the human enzyme. In fact, the reduced destabilizing effect shown by ADA in comparison with that of wt HP-RNase is in accordance with the considerable reduction of its activity against dsRNA.

It is worth pointing out that, under similar assay conditions, human nptRNases 2 and 3 (the so-called EDN and ECP), although highly basic proteins, were found to be incapable of destabilizing the secondary structure of the polydeoxynucleotide and were also totally inactive on dsRNA (*36*, *62*). In RNase 2 indeed the distribution of basic amino acid residues on the protein surface (*63*) is very different from that of RNase 1 (*35*). Therefore, both the dsRNA helix-destabilizing and cleaving efficiencies are peculiar features of some mammalian ptRNases and actually do not appear to be linked to the net charge of the protein. Rather, they seem to arise from the multiplicity of electrostatic interactions between specifically located basic residues of the RNase molecule and the phosphates of the substrates. The recent results obtained by Zhang et al. (*64*) studying the products of two primate paralogous RNase 1 genes are in agreement with our hypothesis. They found that one of these pancreatic RNases had a very low activity on dsRNA. This was related by the authors to amino acid substitutions that involve charge changes. In particular, four of them (Arg-4, Lys-6, Arg-32, and Arg-39) coincide with the residues shown in Table 1 and include the Arg residue at position 4, which was mutated in this study.

Our observations are supported by the models shown in Figure 8. It appears that the surface electrostatic potential of the triple mutant ADA (right) is remarkably different from that of the wild-type enzyme (left). In particular, by substituting the two basic residues Arg-4 and Lys-102 of HP-RNase with two alanines, a considerable reduction resulted of the positive potential of the phosphate binding region. In addition, the positive side chain of Arg-39, that in wt HP-RNase points at the P0−P1 subsites (see Figures 1 and 8A), in the triple mutant ADA (Figure 8 B) appears

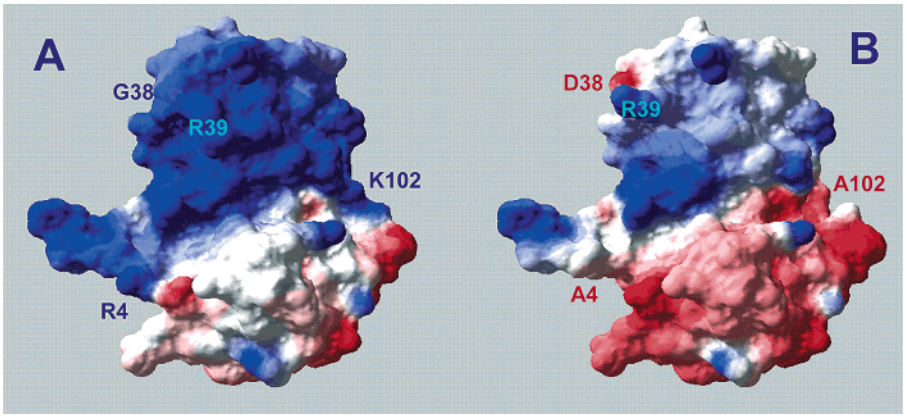

FIGURE 8: Surface electrostatic potentials of HP-RNase (A) and its triple mutant R4A/G38D/K102A (B). The figure was prepared with the program SWISS-Pdb Viewer (*41*).

to be oriented upward by interacting with the negative charge introduced at position 38.

Conclusions and Implications. Our results demonstrate that the remarkable activity shown under physiological salt conditions by HP-RNase against dsRNA is due to the presence of additional noncatalytic basic residues located on the periphery of the substrate binding site of the enzyme. These residues would cooperatively increase the positive electrostatic potential of that region, improving the multiple Coulombic interactions between the RNase molecule and the phosphates of the dsRNA substrate. As a consequence, HP-RNase, given its preferential binding for spontaneously exposed single-stranded sequences of the substrate, can destabilize and then degrade the secondary structure of dsRNA more efficiently than other mammalian RNases.

The data presented here have identified the structural determinants of the enzyme, which as a result of suitable evolutionary processes generate the enhanced activity of HP-RNase against poly(A) and dsRNA. They do not, however, answer the question as to why evolution created in humans an enzyme endowed with such ability. At any rate, the great catalytic versatility of this ribonuclease, together with its wide extracellular distribution ascribable to its specific expression by blood vessel endothelial cells (*34*), might have important physiological implications. Thus, HP-RNase most likely evolved to perform, besides digestive functions, other important extracellular roles in blood serum and body fluids. For example, it may be involved, as already suggested (*36*), in antiviral host defense, a proposal supported by the finding that HP-RNase associated with β-core preparations of human chorionic gonadotropin has anti-HIV replication effects (*65*). HP-RNase might also act as an extracellular RNA scavenger contributing to the normalization of serum viscosity. Moreover, the surprising ability of dsRNA to cross cell membranes and produce a potent RNA interference (*66*) suggests that HP-RNase may be involved in nonspecific response to extracellular pathogenic dsRNA.

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