Origin of the Catalytic Activity of Bovine Seminal Ribonuclease against Double-Stranded RNA†

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ABSTRACT: Bovine seminal ribonuclease (RNase) binds, melts, and (in the case of RNA) catalyzes the hydrolysis of double-stranded nucleic acid 30-fold better under physiological conditions than its pancreatic homologue, the well-known RNase A. Reported here are site-directed mutagenesis experiments that identify the sequence determinants of this enhanced catalytic activity. These experiments have been guided in part by experimental reconstructions of ancestral RNases from extinct organisms that were intermediates in the evolution of the RNase superfamily. It is shown that the enhanced interactions between bovine seminal RNase and double-stranded nucleic acid do not arise from the increased number of basic residues carried by the seminal enzyme. Rather, a combination of a dimeric structure and the introduction of two glycine residues at positions 38 and 111 on the periphery of the active site confers the full catalytic activity of bovine seminal RNase against duplex RNA. A structural model is presented to explain these data, the use of evolutionary reconstructions to guide protein engineering experiments is discussed, and a new variant of RNase A, **A**(Q28L K31C S32C D38G E111G), which contains all of the elements identified in these experiments as being important for duplex activity, is prepared. This is the most powerful catalyst within this subfamily yet observed, some 46-fold more active against duplex RNA than RNase A.

Contemporary biological chemistry seeks to understand the relation between the sequences of proteins and their physical and catalytic properties. One approach is to rationally manipulate proteins using site-directed mutagenesis technology. Analogous manipulation has, of course, been achieved over the past 2 billion years by evolutionary processes, although by random variation and selection, not by rational design (*pace* creationism). The recruitment of established proteins to perform new functions has been documented in dozens of cases (*1*), and the comparison of homologous protein sequences has been used as a tool to guide protein engineering from its inception (see, for example, refs *2* and *3*). Nevertheless, the explosion of the genome sequence databases (see, for example, ref *4*) has dramatically improved our ability to reconstruct with rigor the historical events by which evolution has yielded the diversity of proteins found on modern earth (*5*, *6*). Thus, it remains timely to ask how a deeper understanding of natural "protein engineering" can help the protein engineer in the laboratory.

The ribonuclease (RNase)[1] superfamily (*7*−*10*) is a well-known system for studying molecular evolution (*11*, *12*). In vertebrates, a gene encoding a single RNase near the time when amphibians and mammals diverged has undergone duplication at least six times, giving rise to RNase variants expressed in many different tissues. The most recent duplications of these separated digestive (*13*) and seminal (*14*) RNases in bovids about 35 million years ago (*15*, *16*). BS RNase comprises ca. 2% of the total amount of protein in bovine seminal plasma (*17*). It is a dimer and has potent antispermatogenic (*18*), immunosuppressive (*19*−*23*), and cytotoxic activities (*24*−*26*), all largely missing from pancreatic RNase A.

Evolutionary processes have also altered the catalytic properties of these RNases. For example, bovine seminal RNase catalyzes the hydrolysis of duplex RNA at physiological salt concentrations (*27*−*30*). Pancreatic RNase has essentially no catalytic activity against duplex RNA under the same conditions. It is not known whether the catalytic activity of bovine seminal RNase against duplex nucleic acids relates to its power as an immunosuppressive and cytotoxic agent, nor is it known what relevance catalytic activity against duplex nucleic acids, immunosuppressivity, or cy-

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[1] Abbreviations: BS, bovine seminal; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; poly(A), polyadenylic acid; poly[dA-dT]·poly[dA-dT], polydeoxyadenylic−thymidylic acid (double-stranded alternating copolymer); poly(A)·poly(U), polyadenylic−polyuridylic acid (double-stranded homopolymer); poly(U), polyuridylic acid; RNase, ribonuclease; RNase A, bovine pancreatic ribonuclease; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; UpA, uridylyl-(3′→5′)-adenosine.

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tostatic activity has to the biological function of BS RNase. Therefore, developing an understanding of the structural origin of the interaction of bovine seminal RNase with duplex oligonucleotides is an important step toward understanding first these biological activities and ultimately the physiological function of seminal RNase itself.

An explanation for the catalytic differences must, of course, reside in the 23 amino acid substitutions separating the seminal and pancreatic homologues. These substitutions, however, might confer catalytic activity against duplex RNA by any number of mechanisms. For example, one hypothesis suggests that the dimeric structure of bovine seminal RNase is alone satisfactory to account for its ability to bind and hydrolyze duplex RNA (*27*) and DNA/RNA hybrids (*30*). This hypothesis is supported by studies of RNase A lyophilized with acetic acid (*31*) to create an artificially dimerized RNase A, which shows a 4−8-fold increased catalytic activity at physiological salt concentrations toward duplex RNA (*28*) and DNA/RNA hybrids (*32*). It is conceivable that the 23 amino acid substitutions separating seminal and pancreatic RNase in ox might confer catalytic activity against duplex RNA simply by causing the RNase to dimerize.

A covalently joined dimeric structure is not, however, necessary for catalytic activity against double-stranded RNA observed in some RNases (*29*). Catalytic activity equal to (and sometimes surpassing) that seen in bovine seminal RNase is observed in many RNases that cannot form a covalent dimer, in particular in human "seminal" RNase (*33*). Therefore, a second hypothesis has been advanced to explain catalytic activity of various RNases against duplex RNA, a hypothesis based on the observation that a number of natural ribonucleases with higher isoelectric points also have elevated double-stranded activity. In this hypothesis, catalytic activity against duplex RNA has been interpreted as being related to the number of positive charges presented by the RNase molecule to its substrate (*34*−*36*). Exceptions to this rule are the two basic proteins, human nonsecretory ribonuclease and human eosinophil cationic protein (ECP), which are exceptionally basic members of the ribonuclease superfamily with little catalytic activity against duplex RNA (*36*, *37*). Bovine seminal RNase, however, is archetypal of a basic RNase homologue. In BS RNase, Lys residues replace Gln-55, Asn-62, Tyr-76, and Asn-113 in pancreatic RNase and Arg replaces Ser-80. The pattern of glycosylation of ribonucleases is also believed to influence catalytic activity against duplex RNA (*36*, *38*−*40*).

We report here a systematic study of the relationship between sequence in a RNase and its ability to bind, melt, and hydrolyze duplex RNA. This work relies in part on the experimental reconstruction of ancient pancreatic RNases from ancestral mammals, living as long ago as 40 million years, within the order Artiodactyla (*6*), which includes the pigs and hippopotamuses, camels and llamas, deer, goats, pronghorn antelope, giraffes, gazelles, and oxen (Figure 1). We show that elevated catalytic activity against duplex RNA is most likely a primitive trait of ancient RNases. This primitive trait was further enhanced in the seminal RNase family following its divergence ca. 35 million years ago. Part of this enhancement is due to the evolution of a dimeric structure in the seminal RNase lineage. In contrast, the basic residues introduced into the seminal RNase family did not enhance the ability of BS RNase to interact with duplex oligonucleotides. However, replacement of Asp-38 by Gly and replacement of Glu-111 by Gly, neither obviously involved in catalysis (based on inspection of a crystal structure), are critical to creating the full catalytic activity of bovine seminal RNase against duplex oligonucleotides.

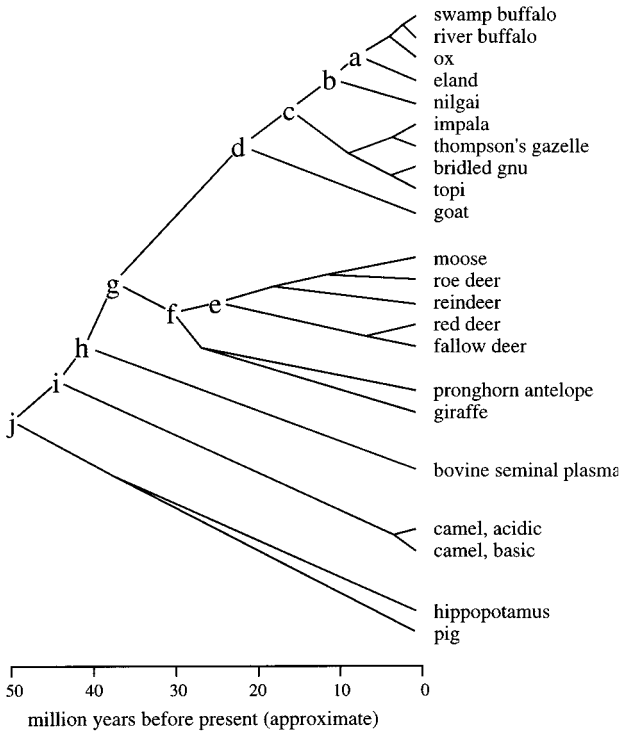

FIGURE 1: Evolutionary tree of artiodactyl ribonucleases. The time scale is approximate. Lowercase letters at the vertexes of the tree indicate the ancestral sequences reconstructed in this work (see Table 1).

MATERIALS AND METHODS

Poly(A) and poly(U) were obtained from Boehringer Mannheim, and poly[dA-dT]·poly[dA-dT] was purchased from Sigma; the other chemicals were of the same quality as described in the preceding paper (*41*). Either recombinant RNase A or RNase A purchased from Boehringer Mannheim was used. Bovine seminal RNase was expressed as described elsewhere (*42*). The computer model was created with the program HyperChem 4.5 (Hypercube).

Construction of Mutants. The construction of the mutant RNase genes was carried out following three different mutagenesis techniques: The modular (or "cassette") mutagenesis technique that was used for the ancient ribonucleases **a**, **b**, **c**, **d**, **g**, $\mathbf{i_1}$, $\mathbf{i_2}$, and $\mathbf{j_1}$[2] and basic surface mutants **A**(Q55K N62K A64T Y76K S80R E111G N113K),[2] **A**(Q55K N62K A64T), **A**(Y76K S80R), and **A**(E111G N113K) is described elsewhere (*12*, *43*−*47*).

The ancient ribonucleases **e**, **f**, $\mathbf{h_1}$, $\mathbf{h_2}$, $\mathbf{j_2}$ (Table 2) and $\mathbf{h_1}$G38D were constructed using the Bio-Rad M13mp19 phage system. The gene for RNase A (*12*) was cloned into M13mp19 using *Eco*RI and *Bam*HI as restriction enzymes. The variants were obtained by the method of Kunkel (*48*). For expression of the protein, the genes were cloned into the expression vector pUN-RNase as described in the preceding paper (*41*).

The mutants **A**(D38G), **A**(D38N), **A**(D38S), **A**(E111G), **A**(D38G E111G), **A**(K31C), **A**(S32C), **A**(K31F S32C)

A(K31C S32C), **A**(Q28L K31C S32C), **A**(A19P Q28L K31C S32C), **A**(K31C S32C D38G E111G), and **A**(Q28L K31C S32C D38G E111G) were constructed using the Muta-Gene Phagemid in vitro mutagenesis kit (version 2) from Bio-Rad, following procedures recommended by the manufacturer. The synthetic RNase A gene (*12*) was cloned in the polylinker-site of pCYTEXP1 (Medac) behind a λ promoter using the *Xho*I and *Bam*HI restriction enzymes. This vector also encodes a temperature-sensitive λ repressor. Construction of BS RNase variants **S**(C31K C32S) and **S**(C31K C32S G38D) was carried out as described elsewhere (*42*). Oligonucleotides were either prepared by automated solid-phase synthesis (Applied Biosystems) or purchased from King's College, London, U.K., or from Microsynth, Windisch, Switzerland. The presence of the desired mutation and the lack of second-site mutations were confirmed by total sequence analysis.

Isolation of Mutant Proteins. Mutant proteins were reconstituted and purified as described in the preceding paper (*41*). This procedure was satisfactory for most variants of RNase A prepared here. However, variants having the dipeptide Arg-Lys at positions 33 and 34 or Lys-Arg at positions 31 and 32 were cleaved between the two basic residues (shown by Edman degradation) when refolded from solutions where urea (8−10 M) was the denaturant. This cleavage was not observed when guanidine hydrochloride (6−8 M) was used as denaturant. Adding urea under the same conditions to a solution of the purified RNase variants did not result in cleavage (*49, 50*). These results are consistent with the observation that the lon$^-$ cell line (*51*) of *E. coli* yields inclusion bodies containing an endoprotease (OmpT) stable to urea but not to guanidine hydrochloride that cleaves between adjacent basic residues (*52*).

Therefore, an alternative procedure was developed for isolating these mutants. The pellet from the centrifugation of the lysed cells was resuspended in 10 times the pellet's weight (approximately 130 mL) of guanidine hydrochloride buffer (6−8 M guanidine hydrochloride, 20 mM ammonium acetate, 500 mM 2-mercaptoethanol, 1 mM PMSF, and 0.02% NaN_3). The inclusion bodies were dissolved by vigorous vortexing and stirring for 30−60 min at room temperature. The solubilization was completed by a 30 min incubation in a 30 °C shaker. After centrifugation (7000*g*, 30 min, 30 °C) the supernatant was diluted with 10 volumes of redox buffer (100 mM NaCl, 50 mM Tris, pH 8, 5 mM EDTA, 10 mM reduced glutathione, 1 mM oxidized glutathione, 1 mM PMSF, and 0.02% NaN_3) and the proteins were refolded for 6−12 h at 4 °C. The solution was centrifuged (7000*g*, 30 min, 4 °C), and the supernatant was dialyzed against NH_4OAc buffer (20 mM, with 0.02% NaN_3, pH 8). A small amount of precipitate was removed by centrifugation (7000*g*, 4 °C, 10 min), and the pH of the supernatant was adjusted to 6.8. Ion-exchange and affinity chromatography were carried out as described in the preceding paper (*41*).

The molar extinction coefficient of RNase was assumed to be 9800 (*34*). For variants where a Tyr was replaced by a nonaromatic amino acid, the extinction coefficients at 278 nm were reduced by 17%, to reflect the reduction by one-sixth of the number of tyrosine residues in the protein (*47*).

Kinetic Assays. Steady-state kinetics were performed with UpA (Sigma) as a substrate (*53*). Poly(A) and poly(U) (Boehringer Mannheim) were combined to yield double-stranded poly(A)·poly(U) following the procedure of Libonati and Floridi (*27*). Poly(A) and poly(U) (1 mg each) were separately dissolved in pure water (1 mL). The solutions were mixed and incubated at room temperature until the absorbance at 260 nm became stable. This mixture was applied to a G-200 column (superfine; Pharmacia) preequilibrated in Tris-HCl buffer (10 mM, pH 7.3, with 150 mM NaCl and 2 mM $MgCl_2$) and eluted. A fraction (2 mL) showing the highest absorption, corresponding to double-stranded oligonucleotides of ca. 1000 base pairs, was used as a substrate for the RNase variants. The specific activities were defined in absorption units and were calculated as done by Libonati and Floridi (*27*) from the initial slope, and expressed as an increase in A_{260} per minute per milligram of protein with 30 μg/mL of poly(A)·poly(U) at 25 °C.

Assays using poly(U) as a substrate were carried out in NaOAc buffer (10 mM, pH 5.0, with 150 mM NaCl). The specific activities were calculated from the initial rates reflected in the slope of the linear part of the recordings and were expressed as an increase in absorbance at 260 nm per minute per milligram of protein with 20 μg/mL poly(U) at 25 °C.

Binding of RNase Variants to Duplex DNA. The melting of duplex poly(dA-dT)·poly(A-T) (13 μg/mL, Boehringer Mannheim) in the presence of RNase variants (13.1 μg/mL) was examined in the buffer used for measuring catalytic activity against duplex RNA but with 50 mM NaCl to permit binding to be observed (*40*). Measurements were obtained in a temperature range of 30−75 °C in a sealed cuvette (1.5 mL, filled to the top).

Reconstruction of Ancient Sequences. Maximum likelihood reconstructions for ancient sequences were made using DARWIN (*5*). Services of the DARWIN package are available by electronic mail (cbrg@inf.ethz.ch) or on the World Wide Web (URL http://cbrg.inf.ethz.ch/) (*54*).

RESULTS

Bovine seminal RNase has additional basic residues at positions 55, 62, 76, 80, and 113 when compared with pancreatic RNase A. Together, these form a "cationic surface" on the protein that is reinforced by the loss of an anionic side chain at position 111, where Glu in RNase A is

[2] Variants of RNase A and seminal RNase are designated first by a boldface letter, indicating the type of RNase type that served as the starting point for the mutation (**A** = RNase A; **S** or BS = bovine seminal RNase), with the amino acid replacements indicated within the parentheses using the one-letter code. The ancestral RNase variants designated **a**−**j**$_2$ refer to vertexes in the evolutionary tree shown in Figure 1 and represent the following mutants: **a** = **A**(A19S L35M N103K), **b** = **A**(A19S L35M K37Q N103K), **c** = **A**(T3S A19S K37Q N103K), **d** = **A**(T3S A19S K37Q N103E), **e** = **A**(T3S S15P A19S K31Q K37Q S59F T70S Y76N T78A S80H A96V N103E), **f** = **A**(T3S A19S K37Q N103E), **g** = **A**(T3S A19S K37Q Y76N S80R N103E), **h**$_1$ = **A**(T3S S16G T17S A19S A20S N34K L35M K37Q D38G A64T Y76N S80R A102V N103E), **h**$_2$ = **A**(T3S S16G A19S A20S N34K L35M K37Q D38G A64T Y76N S80R A102V N103E), **i**$_1$ = **A**(T3S A6E S16G T17S A19S A20S S22N S32R N34K L35M K37Q D38G A64T Y76N S80H T100S A102V N103Q), **i**$_2$ = **A**(A6E S16G T17S A19S A20S S22N S32R N34K L35M K37Q D38G A64T Y76N S80H T100S A102V N103Q), **j**$_1$ = **A**(T3S A6K S16G T17S A19S A20S S22N S32R L35M K37Q D38G A64T Y76N S80H T100S A102V N103Q), and **j**$_2$ = **A**(T3S A6K S16G T17S A19S A20S S22N S32R L35M K37Q D38G A64T Y76N S80H T100S A102E N103Q).

Table 1: Catalytic Activity of Hybrids of Bovine Seminal and Pancreatic RNase[a]

	poly(A)·poly(U)[b] relative to RNase A	poly(U) (% of RNase A)[c]	UpA k_{cat}/K_m (% of RNase A)
RNase A[d]	1.0	100	100
RNase A[e]	1.0	97	90
RNase A lyophilized from HOAc dimer	7.0	nd	nd
A(S32C)	3.1	109	59
A(K31C)	2.0	96	51
A(K31F S32C)	1.1	125	91
A(K31C S32C)	2.1	110	140
A(Q28L K31C S32C)	8.0	nd	102
A(A19P Q28L K31C S32C)	8.0	nd	106
A(Q55K N62K A64T Y76K S80R E111G N113K)	3.8	nd	72
A(Q55K N62K A64T)	0.9	130	26
A(E111G N113K)	3.6	90	104
A(E111G)	3.6	100	104
A(D38G)	5.2	93	110
A(D38G E111G)	10.0	93	102
A(K31C S32C D38G E111G)	27.6	93	88
A(D38N)	3.0	100	97
A(D38S)	5.0	94	91
A(Q28L K31C S32C D38G E111G)	46.4	113	94
S(C31K C32S G38D)	3.0	113	79
S(C31K C32S)	11.6	54	57
bovine seminal RNase[d]	25.0	39	46

[a] All data reported with standard error of ±10%. nd = not determined. [b] Prepared and purified as described in Materials and Methods; 30 μg/mL. [c] At 20 μg/mL. [d] Expressed in *E. coli*. [e] From Boehringer Mannheim.

replaced by Gly in BS RNase. Ala-64 is also substituted by Thr on the same surface. Although this substitution does not change the charge of the protein, the substitution is included in several of the "cationic surface variants" examined here. Additional cationic residues are conserved at positions 61 and 66 in both seminal and pancreatic RNase. Thus, the cationic surface of BS RNase has a total of six positive charges without any compensating negative charges. Several authors have suggested that this cationic surface is important to catalytic activity against duplex RNA observed in bovine seminal RNase (*34*−*36*).

To learn whether the cationic surface is responsible for the increased (compared with RNase A) catalytic activity of BS RNase against duplex RNA, three variants were prepared in a first round of mutagenesis: **A**(Q55K N62K A64T), **A**(E111G N113K), and **A**(Q55K N62K A64T Y76K S80R E111G N113K). Except for **A**(Q55K N62K A64T), each was within a factor of 2 as active as RNase A against the small molecule substrate UpA (Table 1). This suggests that these RNase variants adopt a native fold. Further, each had catalytic activity against single-stranded poly(U) within a factor of 2 the same order of magnitude as that displayed by RNase A (Table 1).

Variant **A**(Q55K N62K A64T Y76K S80R E111G N113K) was examined in a second round of mutagenesis. Against duplex RNA [poly(A)·poly(U)], this variant had catalytic activity increased 3.6−3.8-fold above that seen with RNase A. This is consistent with the notion that the basic surface confers increased double-stranded activity. Dissection of the basic surface showed, however, that the entire effect could be achieved by substitutions at positions 111 and 113 alone. Thus, the catalytic activity of variant **A**(E111G N113K) against duplex RNA was increased 3.6−3.8-fold over that seen with RNase A, while that of variant **A**(Q55K N62K A64T) was identical within experimental error to that of RNase A. Variant **A**(E111G) was then prepared (which has a charge made more positive by one unit even though it does not introduce a basic residue) and was found also to have 3.6-fold increased catalytic activity against duplex RNA. Thus, the impact of the basic surface on this activity could be effectively localized to a single substitution at position 111, which did not introduce a cationic center into the molecule but rather removed an anionic center. Thus, additional basic residues of seminal RNase do not appear to be a determinant of duplex activity in the RNase family of proteins.

Next, the impact of quaternary structure on catalytic activity against duplex RNA was examined. This work was facilitated by the availability of variants having different quaternary structures (*41*), including the dimers **A**(K31C), **A**(S32C), **A**(K31F S32C), and **A**(K31C S32C). Variants **A**(K31C), **A**(S32C), and **A**(K31C S32C) had catalytic activity against duplex RNA enhanced by a factor of 2−3 above that displayed by monomeric RNase A. With variant **A**(K31F S32C), the enhancement was not statistically significant. Thus, dimers generally have increased activity against duplex nucleic acid compared to monomers, but the increase is small compared with the increase observed in bovine seminal RNase itself.

We then explored the influence of the nature of the dimer on catalytic activity against duplex RNA. In the BS RNase dimer, residues 1−20 [the S-peptide, so-called because it was first prepared by cleaving RNase A with subtilisin (*55*)] are exchanged between the two subunits to yield a "swapped" dimer. In the swapped dimer, the active sites are composite, with the catalytically important His-12 coming from one subunit and His-119 from the other. A variety of mutants were assayed for the extent of swapping using a method involving divinyl sulfone as a cross-linking agent (*41*). No clear correlation was observed between catalytic activity against duplex nucleic acids and the extent of swap over all proteins examined (see Discussion).

Two types of dimer distinguished by two types of intersubunit connectivity have been identified (*41*). The "antiparallel" connectivity has Cys-31 in each subunit joined with Cys-32 in the other, while the "parallel" connectivity joins Cys-31 from one subunit with Cys-31 of the other and Cys-32 from each subunit with Cys-32 from the other. The extent of antiparallel connectivity increased in the series **A**(K31C S32C) (35% ± 20% in the antiparallel disulfide linkage), **A**(Q28L K31C S32C) (75% ± 10% in the antiparallel disulfide linkage), and **A**(A19P Q28L K31C S32C) (75% ± 15% in the antiparallel disulfide linkage). Catalytic activity against duplex activity also increased in this series in this order (Table 1).

An alternative strategy was pursued in parallel to learn how evolution engineered catalytic activity against duplex RNA within the RNase superfamily of proteins. Putative sequences that represent possible intermediates in the evolution of RNase within the artiodactyl family over the past 40 million years were obtained from Beintema (personal communication). These were placed on a tree constructed using maximum parsimony tools (*56*−*58*) and inspected for their biological plausibility. After adjustment for information contained in the fossil record (see Discussion), genes

Table 2: Sequence Changes in Reconstructed Ancient Ribonucleases[a]

	RNase	ancestral sequences													
	A	**a**	**b**	**c**	**d**	**e**	**f**	**g**	**h_1**	**h_2**	**i_1**	**i_2**	**j_1**	**j_2**	sem
3	Thr	Thr	Thr	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser*	Thr*	Ser	Ser	Ser
6	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Glu	Glu	Lys	Lys	Ala
15	Ser	Ser	Ser	Ser	Ser	Pro	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser
16	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Gly*	Gly*	Gly*	Gly*	Gly	Gly	Gly
17	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Ser*	Thr*	Ser	Ser	Ser	Ser	Asn
19	Ala	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Pro
20	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ser	Ser	Ser	Ser	Ser	Ser	Ser
22	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Asn*	Asn*	Asn	Asn	Ser
31	Lys	Lys	Lys	Lys	Lys	Gln	Lys	Lys	Lys	Lys	Lys	Lys	Lys*	Lys*	Cys
32	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Arg	Arg	Arg	Arg	Cys
34	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn*	Lys*	Lys*	Lys*	Lys*	Asn	Asn	Lys
35	Leu	Met	Met	Leu	Leu	Leu	Leu	Leu*	Met	Met	Met	Met	Met	Met	Met
37	Lys	Lys	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln
38	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Gly	Gly	Gly	Gly	Gly	Gly	Gly
59	Ser	Ser	Ser	Ser	Ser	Phe	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser
64	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Thr	Thr	Thr	Thr	Thr	Thr	Thr
70	Thr	Thr	Thr	Thr	Thr	Ser	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr
76	Tyr	Tyr	Tyr	Tyr	Tyr	Asn	Tyr	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Lys
78	Thr	Thr	Thr	Thr	Thr	Ala	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr
80	Ser	Ser	Ser	Ser	Ser	His	Ser	Arg*	Arg*	Arg*	His	His	His	His	Arg
96	Ala	Ala	Ala	Ala	Ala	Val	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala
100	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Ser	Ser	Ser	Ser	Thr
102	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Val	Val	Val*	Val*	Val*	Glu*	Val
103	Asn	Lys	Lys	Lys	Glu	Glu	Glu	Glu	Glu	Glu	Gln	Gln	Gln	Gln	Glu

[a] Reconstructed ancient sequences are designated by lowercase boldface letters (Figure 1), with subscripts indicating nodes in the evolutionary tree where more than one alternative sequence was reconstructed. The sequence for bovine seminal RNase is indicated by sem. Only those positions where the ancestral sequences differ from those of contemporary RNase are shown. The ancient sequences were adapted from Beintema et al. (*11*), who calculated them using the maximum parsimony procedure of Fitch (*58*) at the amino acid level. A single modification in the Beintema tree (*11*), which places pig and hippopotamus as a subfamily diverging together from the main lineage (as opposed to having them diverge individually from the main lineage, see Figure 3) resolved several ambiguities in the Beintema−Fitch reconstructions without altering any unambiguously assigned amino acids. Those amino acids marked with asterisks indicate positions where assignment depends on ambiguous parsimony reconstructions or might be changed by plausible reorganization of the tree.

Table 3: Catalytic Activity of Reconstructed Ancient RNases against Duplex RNA

RNase	ancestor of	poly(A)·poly(U) relative to RNase A	poly(U) (% of RNase A)	UpA $k_{cat}/K_m \times 10^6$	k_{cat}/K_m (% of RNase A)
RNase A[a]		1.0	100	5.0	100
RNase A[b]		1.0	97	4.5	90
a	ox, buffalo, eland	1.4	106	6.1	122
b	ox, buffalo, eland, nilgai	1.0	112	5.9	118
c	**b** and the gazelles	0.8	97	4.5	91
d	bovids	0.9	86	3.9	78
e	deer	1.0	77	3.6	73
f	deer, pronghorn, giraffe	1.0	103	3.3	67
g	Pecora	1.0	87	4.6	94
h_1	Pecora and BS RNase	5.2	106	5.5	111
h_2	Pecora and BS RNase	5.2	106	6.5	130
i_1	Ruminata	5.0	96	4.5	90
i_2	Ruminata	4.3	80	5.2	104
j_1	Artiodactyla	4.6	73	3.7	74
j_2	Artiodactyla	2.7	51	3.3	66
h_1(G38D)		1	94	4.9	98
A(D38G)		5	103	5.5	110
bovine seminal RNase		22	39	2.3	46

[a] Expressed in *E. coli*. [b] From Boehringer Mannheim.

encoding these hypothetical ancestral proteins were prepared and expressed (Table 2). In several cases when significant ambiguities were found in the ancient reconstructions, alternative ancestral RNases were prepared (see Discussion). Finally, a parallel set of reconstructions were obtained using a maximum likelihood method (*59*) implemented within the DARWIN system (*5*).

The catalytic activity against duplex RNA of the hypothetical ancestral sequences reconstructed for organisms living more recently than ca. 40 million years was essentially the same as that observed with contemporary pancreatic bovine RNase A (Table 3). In the more ancient reconstructed proteins (ancestors **h_1**, **h_2**, **i_1**, **i_2**, **j_1**, and **j_2**; see Figure 1), however, this catalytic activity was enhanced by a factor of 3−5. Dissection of ancestor **h** was assisted by the experimental fact that the digestive RNase from pronghorn antelope has 7-fold increased catalytic activity against duplex RNA (*15*). Pronghorn and bovine seminal RNase differ at nine positions in their primary structures. The only amino acid residue common to all three sequences (BS RNase, prong-

horn RNase, and ancestor **h**) but absent in RNase A is Gly-38. Therefore, a variant of RNase A was constructed with Asp-38 replaced by Gly as the only amino acid substitution. This mutant had essentially identical double-stranded activity as ancestor **h**, 5 times higher than RNase A.

To confirm that Gly-38 in the ancestral protein was the key to the enhanced catalytic activity of this ancestor against duplex RNA, Gly-38 was removed from the ancient protein and replaced by Asp, the amino acid found in the descendent proteins. This created the variant $\mathbf{h_1}$(G38D), whose catalytic activity against duplex RNA substrates was restored to that of RNase A and the other reconstructed RNases descendent from $\mathbf{h_1}$. This implies that essentially all of the catalytic activity found in ancestral RNase **h** (and earlier ancestors) can be ascribed to the presence of Gly at position 38.

Identification of residue 38 as an element important to the activity of bovine seminal RNase against double-stranded RNA prompted an examination of substitutions other than Gly at this position, in particular, substitutions that may have been introduced during natural selection in other RNases from other organisms. Asn is present at position 38 in the pancreatic RNase from nilgai, whereas Ser is found in impala (*60*). Asp-38 has also been replaced both by Asn and Ser during the divergent evolution of RNase. The RNase variants **A**(D38N) and **A**(D38S) had increased double-stranded activity, although the increase was only about half that observed when Asp-38 was replaced by Gly (Table 1). This suggested that while a portion of the increase in catalytic activity against duplex RNA in the D38G mutant is due to the removal of the charge, another portion arises due to the removal of the side chain.

These results imply that Gly-111, Gly-38, Cys-31, and Cys-32 in BS RNase account entirely for the enhanced catalytic activity of bovine seminal RNase against duplex RNA, assuming that the effects of these substitutions are additive. To test this model, these residues were introduced in pairs into RNase A. First, substitutions at positions 38 and 111 were combined to create **A**(D38G E111G). This variant had greater catalytic activity than either the **A**(D38G) or **A**(E111G) variants, 10-fold higher than that observed with RNase A but still a factor of 2 less than that observed in bovine seminal RNase. This suggested that the contribution to this catalytic activity made by a dimeric structure was independent of the contribution made by Gly-38 and Gly-111.

Variant **A**(K31C S32C D38G E111G) was then created. This protein was a dimer, as expected (*41*). It had a very high catalytic activity against duplex RNA, some 30 times higher than that of RNase A. This activity is somewhat higher than the corresponding activity of bovine seminal RNase itself. Judging by cross-linking with divinyl sulfone (*41*), the protein was found to have only a small (ca. 20%) amount of S-peptide swap. While not ruling out the possibility that some other combination of residues not examined in this work might also have high catalytic activity against duplex RNA, these reconstructions provide an accounting for the high catalytic activity of BS RNase itself in terms of just four residues, Cys-31, Cys-32, Gly-38, and Gly-111, whose effects are roughly additive.

To test this model, reciprocal mutants were prepared in bovine seminal RNases. Cys-31 and Cys-32 were replaced in bovine seminal RNase by Lys and Ser to yield the variant

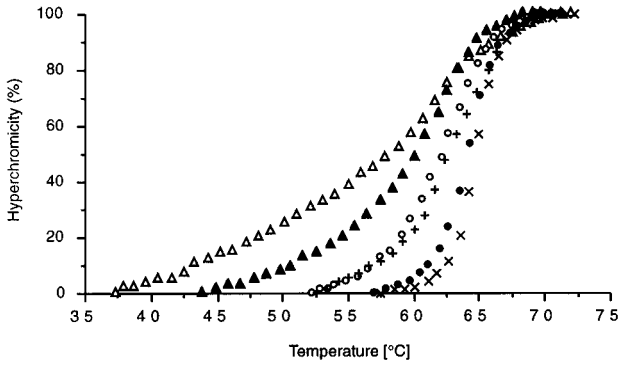

FIGURE 2: Melting of poly[dA-dT]·poly[dA-dT] in the presence of RNase variants. △, A(K31C S32C D38G E111G); ▲, A(D38G E111G); ○, A(D38G); +, A(E111G); ●, RNase A; ×, no protein.

S(C31K C32S). This monomer, lacking the intermolecular disulfide bridges found in bovine seminal RNase, showed a 12-fold increased catalytic activity against duplex RNA relative to RNase A, corresponding to a 2-fold decrease in catalytic activity relative to BS RNase, consistent with a small but significant role of dimeric structure in catalytic activity against duplex nucleic acids. Thus, this variant had a catalytic activity against duplex RNA essentially the same as that displayed by **A**(D38G E111G). To analyze further the role of position 38 in the context of BS monomer, variant **S**(C31K C32S G38D) was constructed. The catalytic activity against double-stranded RNA was further reduced to 3 times that of RNase A.

To explore the mechanism by which RNases might achieve catalytic activity against duplex RNA, the ability of RNase variants to bind and melt duplex DNA was measured (Figure 2). An excellent correlation was found between the affinity of a RNase for duplex DNA and its ability to hydrolyze double-stranded RNA. This suggests that an RNase achieves increased ability to hydrolyze duplex RNA by acquiring improved capacity to bind to and melt duplex oligonucleotides.

Catalytic activity against duplex RNA is often a function of conditions as well as the structure of the catalyst. In particular, the concentration of salt greatly influences the capability of a RNase to catalyze the hydrolysis of duplex RNA (*36*, *40*). As the equilibrium between duplex and monoplex oligonucleotides is also influenced by the ionic strength, it is difficult to separate the two factors. As a starting point for further interpretation, the effect of ionic strength on the catalytic activity of **A**(D38G), **A**(E111G), **A**(D38G E111G), and **A**(K31C S32C D38G E111G) was examined in Tris-HCl buffer (pH 7.3) at different salt concentrations (Table 4). In general, the catalytic activity of the RNase variants against duplex RNA increased with decreasing salt concentration, both with and without magnesium. This is interpreted as a correlate of the well-known instability of duplex RNA at lower dielectric constants and is consistent with a model that suggests that a RNase must melt the duplex structure before it can catalyze the hydrolysis of one strand.

Many workers have reported that catalytic activity is a function of salt concentration. A bell-shaped curve is observed with a maximum shifted to higher salt concentrations with more basic RNase variants (*36*, *61*, *62*). As the data in Table 4 show, a similar effect might be inferred in

Table 4: Activity of RNase Variant against Poly(A)·Poly(U) as a Function of Ionic Strength[a]

	[NaCl] (mM)					
	0	15	45	90	150	200
(a) Buffer: Tris-HCl (10 mM, pH 7.3)						
RNase A[b]	652	360	190	32	2	1
A(E111G)	594	360	190	80	4	3
A(D38G)	812	755	250	160	8	4
A(D38G E111G)	894	740	250	160	9	7
A(K31C S32C D38G E111G)	345	245	160	150	60	16
(b) Buffer: Tris-HCl (10 mM, pH 7.3) and $MgCl_2$ (2 mM)						
RNase A[b]	10	6	2		0.5	
A(E111G)	20	19	10		2.2	
A(D38G)	26	26	12		2.3	
A(D38G E111G)	26	34	30		4.6	
A(K31C S32C D38G E111G)	27	34	40		14.3	

[a] Poly(A) and poly(U) (Boehringer Mannheim) were annealed to yield double-stranded poly(A)·poly(U), which was purified by size-exclusion chromatography (G-200, superfine; Pharmacia) (*27*). This was used as a substrate for the RNase variants following the general procedure of Sorrentino et al. (*61*). Specific activities are reported, calculated from the initial rates reflected in the slope of the linear parts of the recordings, and expressed as an increase in absorbance at 260 nm per minute per milligram of protein with 30 μg/mL of poly(A)·poly(U) at 25 °C. [b] Expressed in *E. coli*.

these variants in the presence of Mg^{2+} (2 mM). The effect is, however, too small to be interpreted.

Last, as a hydrophobic residue at position 28 is believed to orient the subunits in the dimer to enforce an antiparallel arrangement of the intersubunit disulfide bonds (*41*), a final variant, **A**(Q28L K31C S32C D38G E111G), was prepared. The disulfide connectivity was then established using the Edman degradation strategy reported by Ciglic et al. (*41*). The variant existed 70% ± 10% in the antiparallel connectivity. It was found to have the highest catalytic activity against duplex RNA yet seen (46 times greater than for RNase A).

DISCUSSION

The protein engineer has the task of generating proteins with different physical and catalytic properties via rational manipulation of protein sequences. As a typical protein has over 100 amino acids and over 1000 heavy atoms (C, N, O, and S), this task is difficult; indeed, fully predictable manipulation of behavior through deliberate manipulation of structure is not even possible in small molecule chemistry. To improve the odds that a protein engineering enterprise will be successful, information in addition to the sequence and the crystal structure is helpful.

Homologous protein sequences have long provided additional information to guide the protein engineer (*1*). Even two homologous sequences with different properties are adequate to formulate hypotheses about which amino acid residues are responsible for different behaviors. Obviously, hypotheses are easiest to devise when two proteins with different behaviors differ at a very small number of positions; one can then systematically make hybrids between the two proteins to identify those residues that are important for the differences in behavior (*63*), a process that has been called "homology scanning" (*64*).

When many amino acid substitutions separate the two sequences, the hybrid approach can be time-consuming. For example, 23 amino acid substitutions separate bovine seminal RNase and pancreatic RNase. Some 7 408 077 hybrids between the two protein sequences are possible. Thus, combinatorial hybrid "space" cannot be examined randomly. Nor is it adequate to do an "orthogonal" search of hybrid space, starting by making all 23 single point replacements, identifying the one with the highest (for example) hydrolytic activity against duplexes, then introducing all 22 of the remaining substitutions individually, finding the one with the highest activity, and repeating this process. Although this approach requires (maximally) 275 hybrids, it assumes that the 23 substitutions have independent impact on behavior in the protein. This is known not to be true in the RNase superfamily (*41*), as well as in many other protein families (*65*, *66*).

A variety of strategies can be used to obtain additional information. First, the properties of naturally occurring homologues might be exploited. This was the approach used by Beintema, Libonati, and Sorrentino (*34*−*36*) to propose that basic residues in a RNase contribute to catalytic activity against duplex RNA. A correlation between the basicity of a RNase and its ability to hydrolyze duplex RNA is theoretically plausible, as additional basic residues on an RNase might create a binding site for the second polyanionic oligonucleotide strand. Beintema, Libonati, and Sorrentino (*34*−*36*) inspected a large number of natural variants within the RNase superfamily for their catalytic activity against duplex RNA, observing a general correlation between catalytic activity and basicity in the protein, consistent with this model.

The hypothesis formulated using this strategy proved, in this case, to be incorrect. No increase in the catalytic activity of BS RNase against duplex RNA could be assigned to any basic residue introduced into BS RNase following its divergence from the pancreatic homologue. The variant **A**(Q55K N62K A64T Y76K S80R E111G N113K), having the entire basic surface of BS RNase, does indeed have increased catalytic activity against duplex RNA. However, the only substitution that evidently has an impact on this activity is the one converting Glu at position 111 to Gly. The loss of the negative charge at position 111 increases the overall charge of the protein by +1 but does not introduce a new basic site. Thus, introduction of basic residues is evidently not an approach to engineer catalytic activity against duplex nucleic acids in these RNases.

A second strategy to identify additional information relies on a crystal structure in light of a structural hypothesis. It seems plausible, for example, that two active sites will help a RNase hydrolyze a substrate with two strands, if only because one active site holds the second strand while the other active site cleaves the first. Thus, it is rational to hypothesize that a dimeric protein will be a better catalyst for the hydrolysis of duplex RNA than a monomeric protein.

The work of Ciglic and colleagues (*41*) provided a range of variants with different quaternary structures useful for testing this hypothesis. Dimeric structure was indeed found to contribute to the ability of a RNase to catalyze the hydrolysis of duplex RNA. Simply joining two RNase monomer units together by a single disulfide bond involving either Cys-31 or Cys-32 enhances catalytic activity of the RNase against poly(A)·poly(U) by a factor of 2−3. The enhancement depends modestly on the context. For example,

the variant **A**(K31F S32C), chosen because Phe is encoded at position 31 by a seminal RNase gene from giraffe (*16*), showed no increase in catalytic activity against duplex RNA. This suggests that the orientation of the subunits in the dimer may also be important for catalytic activity against duplex nucleic acids.

Dimeric structures are known to come in two types, one where the first 20 amino acids (the S-peptide) are exchanged, or swapped, between subunits and the other where they are not (see ref *41* for references). S-peptide swapping does not, however, appear to be a structural feature that determines catalytic activity against duplex RNA. For examples, variant **A**(Q28L K31C S32C) and variant **A**(A19P Q28L K31C S32C) have approximately the same catalytic activity against duplex RNA, even though the second is ca. 80% swapped, while the former is 40% swapped.

The failure of both the "basic surface" hypothesis and the "dimeric structure" hypothesis to together account fully for the increased catalytic activity of BS RNase against duplex oligonucleotides prompted a third strategy for investigation. By applying rules of parsimony to the sequences of contemporary RNase sequences (*58*, *67*), the recent evolutionary history of the RNase family was reconstructed. This allowed us to retrace the steps by which nature has evolved proteins that act on double-stranded nucleic acid (Figure 1). Fourteen ancestral RNases with sequences approximating those presumed to be evolutionary intermediates in the evolution of pancreatic RNases were constructed and studied (Table 2) (*6*). These dated back beyond the putative most recent common ancestor of bovine seminal RNase and RNase A (Figure 1, Table 2). The catalytic activity of these ancient proteins from now extinct organisms against poly(A)·poly(U) was measured (Table 3) (*6*). For most of the recent evolution of RNase A, catalytic activity against duplex oligonucleotides remained low. Going back in geological time, however, an increase in catalytic activity against duplex RNA was observed upon going from ancestral protein **g** (the most recent common ancestor of Pecora, which includes the deer, sheep, and bovids) to ancestral protein **h**, the point where bovine seminal RNase diverges in Beintema's parsimony analysis (*11*) (Table 3).

Between ancestor **h** and ancestor **g**, Gly-38 is replaced by Asp, which is retained in most of the descendent artiodactyl pancreatic RNases. The precise point where this substitution is made in the reconstructed history of the protein depends on the exact nature of the tree (Figure 3) (*68*). Nevertheless, it is clear that the change was made ca. 40 million years ago, near the time when ruminant artiodactyls diverged from nonruminant artiodactyls (*68*). Pronghorn RNase has a Gly at position 38, however, and shows a 7-fold increased catalytic activity against double-stranded nucleic acid (*15*). These observations prompted investigation of the amino acid substitution at position 38, which was found also to be critical for catalytic activity against duplex RNA.

Thus, these three strategies together identified four residues, Cys-31, Cys-32, Gly-38, and Gly-111, that appeared to be sufficient to confer the full catalytic activity against duplex RNA found in seminal RNase. We then asked what mechanistic hypotheses might be formulated to explain why these substitutions have their effect. In the RNase A mechanism, the 2′-hydroxyl group attacks the 3′-phosphate

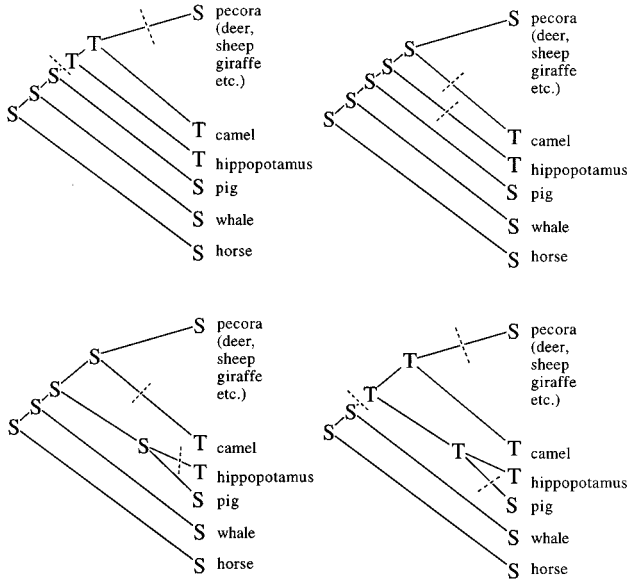

FIGURE 3: Reconstructed amino acids depend on the connectivity of an evolutionary tree, here illustrated in the reconstruction of Thr or Ser at position 3. Nevertheless, it is clear that in this case different trees give only slightly different reconstructions, with no impact on the conclusions drawn from them.

in an "in-line" mechanism (*69*). In this mechanism, the 5′-oxygen of the following nucleoside must lie 180° away from the attacking oxygen (*70*). In a standard A-type double helix adopted by RNA, this geometry is not possible. The 2′-hydroxyl group is not within bonding distance of the 3′-phosphate, and the 5′-oxygen of the following nucleoside is not placed 180° away from the 2′-hydroxyl group.

This implies that to hydrolyze duplex RNA via the RNase A mechanism, the catalyst must locally melt the double helix (*36*, *71*). If this is indeed a step in the catalytic cycle by which these variants achieve catalytic activity against duplex RNA, it should correlate with the capacity of an RNase variant to bind and melt double-helical DNA. The observation that ancestral RNase $\mathbf{h_1}$ binds to cellulose bearing double-stranded DNA in 50 mM NaCl solution, whereas RNase A does not (*50*), suggested that this might be the case.

To explore the relationship between the ability to bind and melt duplex oligonucleotides and the ability to catalyze the hydrolysis of duplex RNA, melting curves were obtained with poly[dA-dT]·poly[dA-dT] duplexes in the presence of different RNase variants. A good correlation exists between the ability of a RNase variant to catalyze the hydrolysis of duplex RNA (Table 1) and its ability to bind and melt duplex DNA (Figure 2). Thus, it is highly likely that duplex melting is the first step in the hydrolysis of duplex RNA catalyzed by the active RNase variants prepared here.

This correlation suggested a structural model to account for the catalytic activity of RNase variants against duplex RNA. Starting with the high-resolution crystal structure of BS RNase provided by Mazzarella and co-workers (*72*) and the structure of RNase A complexed with d(ApTpApApG) (*73*), we built a model for the putative interaction between duplex RNA and RNase joined as a dimer through disulfide bonds having the antiparallel connectivity (Cys-31 from each subunit pairs with Cys-32 from the other). The model made the assumption that both active sites of the dimer bind to an

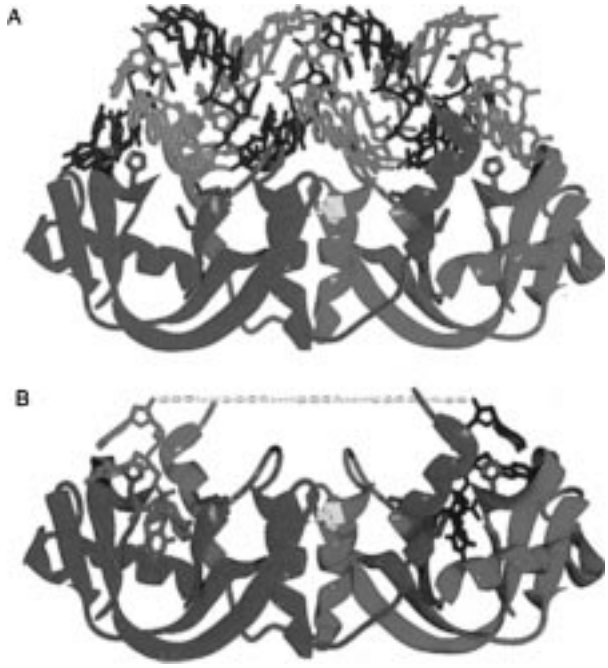

FIGURE 4: Model of the interaction of BS RNase with idealized A-RNA. Amino acid side chains in the active site (His-12, His-119, Lys-41) are marked in green; the intermolecular disulfide bonds (Cys-31/32′ and Cys-32/31′) are colored yellow; amino acid side chains at positions 38, 38′ and 111, 111′ are in black; the A-RNA strands are red and blue. Panel A is a model of the interaction of BS RNase (*72*) with A-RNA. In panel B the crystal structures of two RNase A·d(ApTpApApG) complexes (*73*) are superimposed on the crystal structure of BS RNase (*72*). The computer model was created with the HyperChem 4.5 program (Hypercube Inc.). Images were produced using the MidasPlus program from the Computer Graphics Laboratory, University of California, San Francisco (*75*).

RNA strand at the same time (Figure 4). Accepting standard geometric parameters of RNA (*74*), one plausible model places different strands in the different active sites. This requires an almost 90° bend in the course of the oligonucleotide chain. The nucleoside 5′ to the diester cleavage site is the partner of a base in the complementary strand 16−17 residues away from the nucleoside 5′ to the linkage that would be cleaved in the first active site. Residues 111 and 38 are on the surface of the protein at each end of the active center. In the model, Asp-38 lies near the RNA chain as it passes from one active site to the other. The loss of a negative charge at position 38 might help the dimer to bind and bend duplex RNA.

This model presumes an antiparallel disulfide connectivity between the subunits where Cys-31 from each subunit is joined with Cys-32 from the other. Cleavage of **A**(K31C S32C D38G E111G) with CNBr followed by Edman degradation (*41*) showed that variant **A**(K31C S32C D38G E111G) had 40−60% of the antiparallel intersubunit disulfide connectivity (see ref *41* for a further discussion of the method). As noted above, the ability to form a dimer with the antiparallel intersubunit disulfide connectivity correlates approximately with catalytic activity against duplex RNA in the series **A**(K31C S32C), **A**(Q28L K31C S32C), and **A**(A19P Q28L K31C S32C). This suggests that the major contribution to duplex activity of the **A**(K31C S32C D38G E111G) variant comes from the fraction with the antiparallel disulfide intersubunit connectivity.

Can this insight into structure−function relationships gained by a combination of evolutionary analysis, structural biology, and wet biochemistry assist the protein engineer in designing variants of RNase with high catalytic activity against duplex RNA? From the results reported by Ciglic et al. (*41*), it was concluded that a hydrophobic residue at position 28 enhanced the amount of antiparallel disulfide connectivity. The variant **A**(Q28L K31C S32C D38G E111G) was therefore prepared, as it adds this hydrophobic residue to the residues determined in this paper to be important for duplex activity. The protein has the highest catalytic activity against duplex RNA observed in this series, some 50% higher than that displayed by seminal RNase and 46-fold higher than RNase A. The intersubunit disulfides in this variant are ca. 70% of the antiparallel type. The high catalytic activity of this variant against duplex RNA is attributed to the presence together in one protein of all of the structural features presumed to be important for this behavior: a dimeric structure (regardless of swap), antiparallel intersubunit disulfide connectivity, and glycine residues at positions 38 and 111.

These results show how evolutionary selection (or, now, the protein engineer) might create a RNase with catalytic activity against duplex RNA. They do not, however, answer the "nonclassical" questions, those relating to why evolution chose to undertake this engineering task. To address such questions, we must understand how catalytic activity against duplex RNA in an RNase, or an ability to bind and melt duplex DNA, help an artiodactyl survive, select a mate, and reproduce. In the pancreatic RNases from artiodactyls, it appears as if this catalytic activity has no selected function. Catalytic activity observed in artiodactyl pancreatic RNases is highly variable. In pronghorn, for example (*15*), the activity is 7-fold higher than in ox. Impala and nilgai pancreatic RNases have Ser and Asn, respectively, at position 38 (*60*); these are therefore expected from the results reported here to have ca. 3-fold increased catalytic activity against double-stranded RNA when compared with ox pancreatic RNase. The variability in this behavior across the evolutionary tree suggests that catalytic activity against duplex RNA plays no selectable physiological role in digestive RNases.

This, in turn, suggests that the special properties conferred by Gly-38 on the RNase may themselves have selective value in more ancient RNases and in the seminal RNase branch of the RNase superfamily, where this Gly has been conserved. The fact that the engineer can create a variant of RNase with still *higher* catalytic activity against double-stranded RNA [variant **A**(Q28L K31C S32C D38G E111G)] presents an interesting conundrum. Either a seminal RNase with maximal duplex activity is not the selective optimum for this protein, this activity is not the goal of natural selection, or natural selection is still in the process of optimizing duplex activity. This question will be addressed elsewhere (*42*, *76*).

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REFERENCES

1. Benner, S. A., and Ellington, A. D. (1990) *Bioorg. Chem. Front. 1*, 1−70.
2. Allemann, R. K., Presnell, S. R., and Benner, S. A. (1991) *Protein Eng. 4*, 831−835.
3. Clarke, A. R., Colebrook, S., Cortes, A., Emery, D. C., Halsall, D. J., and Hart, K. W. (1991) *Biochem. Soc. Trans. 19*, 576−581.
4. Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J. F., Dougherty, B. A., et al. (1995) *Science 269*, 496−512.
5. Gonnet, G. H., Cohen, M. A., and Benner, S. A. (1992) *Science 256*, 1443−1445.
6. Jermann, T. M., Opitz, J. G., Stackhouse, J., and Benner, S. A. (1995) *Nature 374*, 57−59.
7. Benner, S. A., and Allemann, R. K. (1989) *Trends Biochem. Sci. 14*, 396−397.
8. Blackburn, P., and Moore, S. (1982) *Enzymes 15*, 317−434.
9. Richards, F. M., and Wyckoff, H. W. (1971) *Enzymes 4*, 647−806.
10. D'Alessio, G., Di Donato, A., Parente, A., and Piccoli, R. (1991) *Trends Biochem. Sci. 16*, 104−106.
11. Beintema, J. J., Fitch, W. M., and Carsana, A. (1986) *Mol. Biol. Evol. 3*, 262−275.
12. Nambiar, K. P., Stackhouse, J., Stauffer, D. M., Kennedy, W. G., Eldredge, J. K., and Benner, S. A. (1984) *Science 223*, 1299−1301.
13. Barnard, E. A. (1969) *Nature 221*, 340−344.
14. D'Alessio, G., and Leone, E. (1963) *Biochem. J. 89*, 7P.
15. Beintema, J. J. (1987) *Life Chem. Rep. 4*, 333−389.
16. Breukelman, H. J., Beintema, J. J., Confalone, E., Costanzo, C., Sasso, M. P., Carsana, A., Palmieri, M., and Furia, A. (1993) *J. Mol. Evol. 37*, 29−35.
17. D'Alessio, G., Floridi, A., De Prisco, R., Pignero, A., and Leone, E. (1972) *Eur. J. Biochem. 26*, 153−161.
18. Matousek, J. (1969) *J. Reprod. Fert. 19*, 63−72.
19. Soucek, J., and Matousek, J. (1981) *Folia Biol. Praha 27*, 334−345.
20. Soucek, J., Hrubá, A., Paluska, E., Chudomel, V., Dostál, J., and Matousek, J. (1983) *Folia Biol. Praha 29*, 250−261.
21. Soucek, J., Chudomel, V., Potmesilova, I., and Novak, J. T. (1986) *Nat. Immun. Cell Growth Regul. 5*, 250−258.
22. Derwenskus, K. H., Sprinzl, M., and Scheit, K. H. (1989) *Biol. Chem. Hoppe-Seyler 370*, 285−292.
23. Tamburrini, M., Scala, G., Verde, C., Ruocco, M. R., Parente, A., Venuta, S., and D'Alessio, G. (1990) *Eur. J. Biochem. 190*, 145−148.
24. Vescia, S., and Tramontano, D. (1981) *Mol. Cell. Biochem. 36*, 125−128.
25. Piccoli, R., Vescia, S., Bridges, S. H., and D'Alessio, G. (1990) *Ital. J. Biochem. 39*, 242−249.
26. Laccetti, P., Portella, G., Mastronicola, M., Russo, A., Piccoli, R., D'Alessio, G., and Vecchio, G. (1992) *Cancer Res. 52*, 4582−4586.
27. Libonati, M., and Floridi, A. (1969) *Eur. J. Biochem. 8*, 81−87.
28. Libonati, M. (1971) *Biochim. Biophys. Acta 228*, 440−445.
29. Libonati, M., Malorni, M. C., Parente, A., and D'Alessio, G. (1975) *Biochim. Biophys. Acta 402*, 83−87.
30. Taniguchi, T., and Libonati, M. (1974) *Biochem. Biophys. Res. Commun. 58*, 280−286.
31. Fruchter, R. G., and Crestfield, A. M. (1965) *J. Biol. Chem. 240*, 3868−3874.
32. Libonati, M., Sorrentino, S., Galli, R., La Montagna, R., and Di Donato, A. (1975) *Biochim. Biophys. Acta 407*, 292−298.
33. De Prisco, R., Sorrentino, S., Leone, E., and Libonati, M. (1984) *Biochim. Biophys. Acta 788*, 356−363.
34. Libonati, M., Furia, A., and Beintema, J. J. (1976) *Eur. J. Biochem. 69*, 445−451.
35. Libonati, M., and Beintema, J. J. (1977) *Biochem. Soc. Trans. 5*, 470−474.
36. Libonati, M., and Sorrentino, S. (1992) *Mol. Cell. Biochem. 117*, 139−151.
37. Sorrentino, S., and Glitz, D. G. (1991) *FEBS Lett. 288*, 23−26.
38. Carsana, A., Furia, A., Gallo, A., Beintema, J. J., and Libonati, M. (1981) *Biochim. Biophys. Acta 654*, 77−85.
39. Beintema, J. J., Blank, A., Schieven, G. L., Dekker: C. A., Sorrentino, S., and Libonati, M. (1988) *Biochem. J. 255*, 501−505.
40. Sorrentino, S., Lavitrano, M., De Prisco, R., and Libonati, M. (1985) *Biochim. Biophys. Acta 827*, 135−139.
41. Ciglic, M. I., Jackson, P. J., Raillard, S. A., Haugg, M., Jermann, T. M., Opitz, J. G., Trabesinger-Rüf, N., and Benner, S. A. (1998) *Biochemistry 37*, 4008−4022.
42. Haugg, M. (1996) ETH Dissertation 11608, Swiss Federal Institute of Technology, Zürich, Switzerland.
43. Wells, J. A., Vasser, M., and Powers, D. B. (1985) *Gene 34*, 315−323.
44. McGeehan, G. M., and Benner, S. A. (1989) *FEBS Lett. 247*, 55−56.
45. Trautwein, K., and Benner, S. A. (1991) *FEBS Lett. 281*, 275−277.
46. Trautwein-Fritz, K. (1991) ETH Dissertation 9613, Swiss Federal Institute of Technology, Zürich, Switzerland.
47. Raillard, S. A. (1993) ETH Dissertation 10261, Swiss Federal Institute of Technology, Zürich, Switzerland.
48. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
49. Jermann, T. M. (1995) ETH Dissertation 11059, Swiss Federal Institute of Technology, Zürich, Switzerland.
50. Opitz, J. G. (1995) ETH Dissertation 10952, Swiss Federal Institute of Technology, Zürich, Switzerland.
51. Chung, C. H., Waxman, L., and Goldberg, A. L. (1983) *J. Biol. Chem. 258*, 215−221.
52. White, C. B., Chen, Q., Kenyon, G. L., and Babbitt, P. C. (1995) *J. Biol. Chem. 270*, 12990−12994.
53. Ipata, P. L., and Felicioli, R. A. (1968) *FEBS Lett. 1*, 29−31.
54. Gonnet, G. H., and Benner, S. A. (1991) in *Computational Biochemistry Research at ETH. Technical Report*, Departement Informatik, Swiss Federal Institute of Technology, Zürich, Switzerland.
55. Richards, F. M., and Vithayathil, P. J. (1959) *J. Biol. Chem. 234*, 1459−1465.
56. Pauling, L., and Zuckerkandl, E. (1963) *Acta Chem. Scand. 17*, S9−S16.
57. Eck, R. V., and Dayhoff, M. O. (1966) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., Ed.) pp 161−169, National Biomedical Research Foundation, Silver Spring, MD.
58. Fitch, W. (1971) *Syst. Zool. 20*, 406−416.
59. Felsenstein, J. (1981) *J. Mol. Evol. 17*, 368−376.
60. Beintema, J. J. (1980) *Biochim. Biophys. Acta 621*, 89−103.
61. Sorrentino, S., Carsana, A., Furia, A., and Doskocil, J. L. M. (1980) *Biochim. Biophys. Acta 609*, 40−52.
62. Libonati, M., Carsana, A., and Furia, A. (1980) *Mol. Cell. Biochem. 31*, 147−164.
63. Nambiar, K. P., Stackhouse, J., Presnell, S. R., and Benner, S. A. (1986) in *Proceedings of the NATO advanced Workshop on Enzymes as Catalysts in Organic Synthesis* (Schneider, M., Ed.) pp 325−339, D. Reidel, Ulm, Germany.
64. Cunningham, B. C., Jhurani, P., Ng, P., and Wells, J. A. (1989) *Science 243*, 1330−1336.
65. Mildvan, A. S., Weber, D. J., and Kuliopulos, A. (1992) *Arch. Biochem. Biophys. 294*, 327−340.
66. First, E. A., and Fersht, A. R. (1995) *Biochemistry 34*, 5030−5043.
67. Fitch, W., and Margoliash, E. (1967) *Science 155*, 279−284.
68. Benner, S. A., Jermann, T. M., Opitz J. G, Stackhouse, J., Knecht L. J, and Gonnet, G. H. (1995) *Nature 377*, 109−110.
69. Westheimer, F. H. (1968) *Acc. Chem. Res. 1*, 70−78.

70. Campbell, R. L., and Petsko, G. A. (1987) *Biochemistry 26*, 8579−8584.
71. Pandit, M. W., and Ramakrishna, T. (1986) *Biochim. Biophys. Acta 867*, 1−8.
72. Mazzarella, L., Capasso, S., Demasi, D., di Lorenzo, G., Mattia, C. A., and Zagari, A. (1993) *Acta Crystallogr. 49*, 389−402.
73. Fontecilla-Camps, J. C., de Llorens, R., le Du, M. H., and Cuchillo, C. M. (1994) *J. Biol. Chem. 269*, 21526−21531.
74. Saenger, W. (1984) *Principles of Nucleic Acid Structure*, Springer-Verlag, New York.
75. Ferrin, T. E., Huang, C. C., Jarvis, L. E., and Langridge, R. (1988) *J. Mol. Graphics 6*, 13−27.
76. Benner, S. A., Trabesinger-Rüf, N., and Schreiber, D. R. (1998) manuscript in preparation.

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