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The Structure of an Engineered Domain-Swapped Ribonuclease Dimer and Its Implications for the Evolution of Proteins toward Oligomerization

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Summary

Background: Domain swapping has been proposed as a mechanism that explains the evolution from monomeric to oligomeric proteins. Bovine and human pancreatic ribonucleases are monomers with no biological properties other than their RNA cleavage ability. In contrast, the closely related bovine seminal ribonuclease is a natural domain-swapped dimer that has special biological properties, such as cytotoxicity to tumour cells. Several recombinant ribonuclease variants are domain-swapped dimers, but a structure of this kind has not yet been reported for the human enzyme.

Results: The crystal structure at 2 Å resolution of an engineered ribonuclease variant called PM8 reveals a new kind of domain-swapped dimer, based on the change of N-terminal domains between the two subunits. The swapping is fastened at both hinge peptides by the newly introduced Gln101, involved in two intermolecular hydrogen bonds and in a stacking interaction between residues of different chains. Two antiparallel salt bridges and water-mediated hydrogen bonds complete a new interface between subunits, while the hinge loop becomes organized in a 3_{10} helix structure.

Conclusions: Proteins capable of domain swapping may quickly evolve toward an oligomeric form. As shown in the present structure, a single residue substitution reinforces the quaternary structure by forming an open interface. An evolutionary advantage derived from the new oligomeric state will fix the mutation and favour others, leading to a more extended complementary dimerization surface, until domain swapping is no longer necessary for dimer formation. The newly engineered swapped dimer reported here follows this hypothetical pathway for the rapid evolution of proteins.

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Introduction

Many proteins have evolved from a monomeric to an oligomeric form, thereby acquiring new abilities or functions. The structural determinants leading to an oligomeric state are, however, difficult to identify because they might be the sum of many small and subtle changes. Their structural analysis can help us in the design of new proteins from existing monomers. One approach to a dimeric association is called domain swapping [1]. It is based on the mutual exchange of an entire domain between each of the molecules of a dimer. Apart from furthering our pursuit of a protein with novel abilities, the structural changes derived from single amino acid substitutions leading to domain swapping may provide a better understanding of the intriguing events of molecular evolution.

Two members of the ribonuclease superfamily provide particularly interesting models for the study of the transition from a monomer to a domain-swapped dimer with special biological actions. Bovine pancreatic ribonuclease (RNase A) [2] is a small, compact monomeric protein of 124 residues and four disulfide bridges, with an N-terminal helix (tail, T) that is centrally positioned between the two lobes of a kidney-shaped structure (body, B). Both domains are connected by a 7 residue hinge loop (Figure 1a). Bovine seminal ribonuclease (BS-RNase), with which RNase A shares 80% of its sequence identity, is a naturally occurring dimer with both subunits covalently linked by two disulfide bonds as well as by noncovalent interactions (Figure 1b; [3]). Two-thirds of the BS-RNase dimers in seminal plasma have their N-terminal domains swapped (M × M quaternary form), whereas in the rest of the dimers each protomer folds onto itself (M = M quaternary form). BS-RNase exhibits distinctive catalytic properties (a strong action on double-stranded RNA and an allosteric regulation), as well as other noncatalytic bioactions (immunosuppressive, aspermatogenic, antiviral, embryotoxic, and cytotoxic on tumor cells) [3]. Upon lyophilization of RNase A monomers from acetic acid solution, this dual ribonuclease system is enriched by the availability of two forms of artificial domain-swapped dimers (Figure 1c; [4]), for which the three-dimensional structure has been recently determined [5, 6]. Although such dimers can degrade RNA, they are not endowed with any of the special bioactions of BS-RNase dimers [7]. Thus, domain swapping cannot be said to be the only cause of special biological activities in the ribonuclease superfamily. A structural comparison suggests that the different abilities shown by the BS-RNase dimer may arise from a particular quaternary structure, which in turn is derived from the different interactions or interfaces between the two protomers. The interface between domains that is present in both the monomer and the do-

Key words: domain swapping; human pancreatic ribonuclease; molecular evolution; protein oligomerization; site-directed mutagenesis; X-ray crystal structure

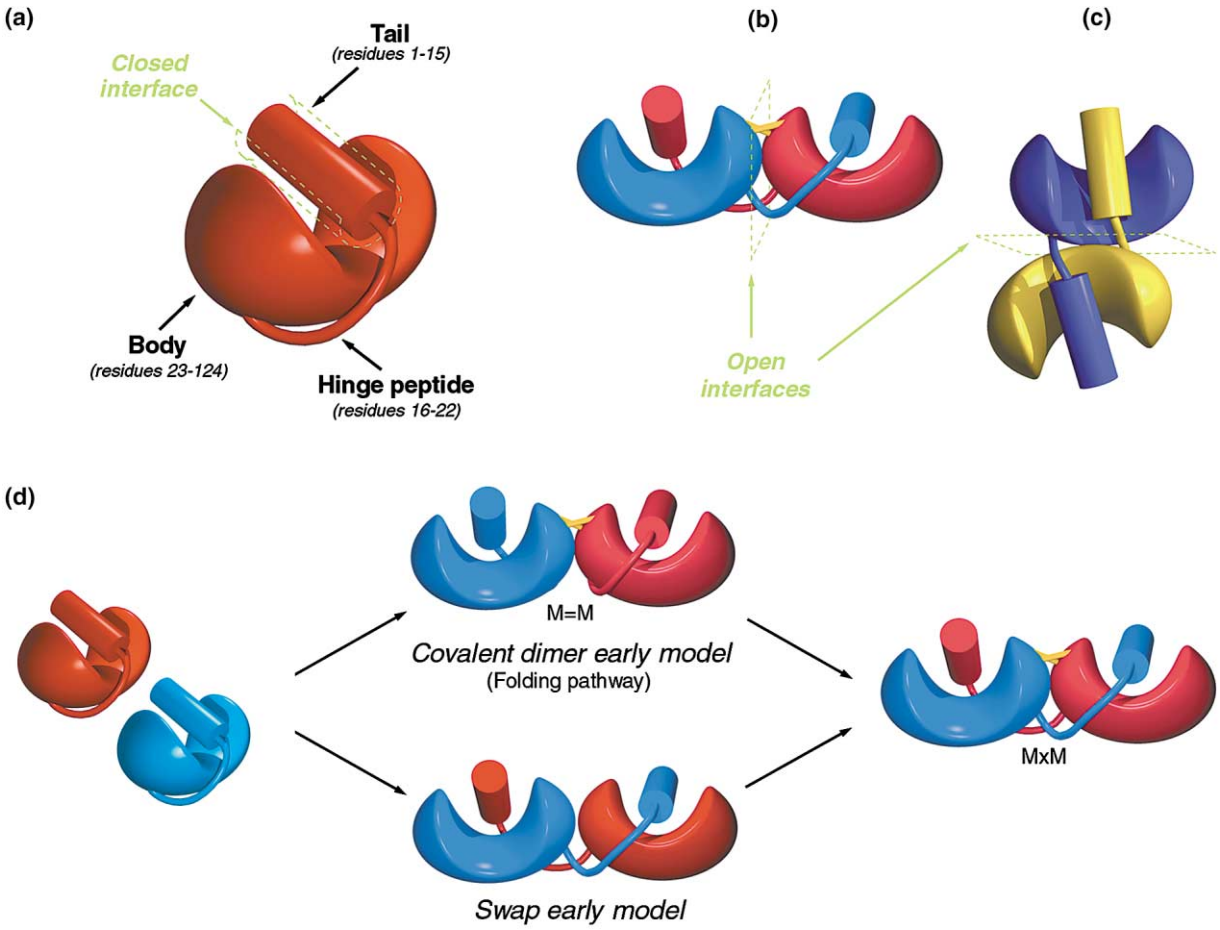


Figure 1. Diagram of Monomeric and Dimeric Ribonucleases

(a) RNase A and its parts.

(b) Natural domain-swapped dimer of BS-RNase.

(c) Artificial domain-swapped minor dimer of RNase A

(d) Two models for the evolutionary process from an RNase monomer to the domain-swapped dimer of BS-RNase (see text). Although present in all the structures, the closed interface is only indicated in RNase A for clarity. Yellow sticks stand for intermolecular disulfide bonds. M = M and M × M are the two possible quaternary conformations of natural BS-RNase (see text).

main-swapped oligomer is called the *closed interface*, whereas the interface found only in the oligomer is called the *open interface* [8].

The folding pathway of dimeric BS-RNase is attractive as a plausible description of the evolutionary process from monomeric to oligomeric proteins in which domain swapping occurs [4]. Both protomers of BS-RNase are folded separately, and in a second step two antiparallel disulfide bonds are formed between two molecules. The result is a covalently linked dimer, with N-terminal domains still nestling against the bodies formed by their own chains. It is from then on that swapping occurs, until the equilibrium of swapped and non-swapped dimers is reached. Such a folding pathway may serve as a *covalent dimer early* model for the evolution of dimers [4], where domain swapping is the last event (Figure 1d, upper pathway). According to this model, dimerization occurs on the basis of strong interactions located at the open interface; these interactions are basically the two antiparallel disulfide bonds (Cys31 and Cys32) and a reduced hydrophobic patch formed by residues Leu28 and Met29 [4]. BS-RNase has been used as a model to obtain dimers of related ribonucleases [9, 10]. This is not surprising since BS-RNase is the only natural ribonuclease dimer endowed with special noncatalytic bioactions, although an alternative model for the evolution of BS-RNase suggests that different approaches are possible.

In contrast to the folding hypothesis, some authors maintain that, given the appropriate partial destabilizing conditions, a noncovalent dimer may be formed by the interchange of noncovalently constrained terminal "domains" [1, 6]. A *swap early* model for the evolution of intertwined dimers is better suited to the notion that swapping is a natural event in any case. The interactions between the exchanged domain and the other subunit are those that can be found in the monomer and form the closed interface. According to this model, dimerization can be induced by even a single amino acid substitution (Figure 1d, lower pathway). The RNase A domain-swapped dimers are extreme examples of such a model. Two dramatically different interfaces are formed in the "mi-

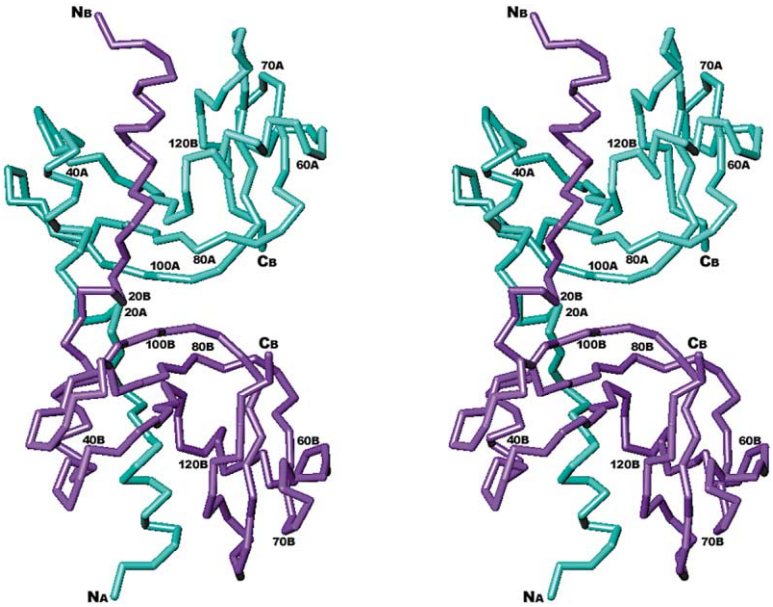


Figure 2. C_α Stereo View of Homodimeric HP-RNase Variant PM8

N-terminal and C-terminal residues have been labeled with an N and a C, respectively. Other residues have been labeled with their number and chain identifier.

nor" (less abundant) and "major" forms of RNase A dimers, where the N-terminal α helices and C-terminal β strands, respectively, are exchanged. It has been suggested that a single mutation that provided stronger interactions along a newly formed open interface would produce a natural and more stable RNase dimer once the swapping had occurred [5].

Human pancreatic ribonuclease (HP-RNase) [11] is a monomeric ribonuclease for which no special bioactions have been reported. Focussing on the basis of the structural and functional determinants of BS-RNase, Piccoli et al. [10] designed and constructed a dimeric variant of HP-RNase that was found to be cytotoxic to several malignant mouse and human cell lines. Here we report the structural characterization of a different dimeric variant of HP-RNase, called PM8, that strongly supports the *swap early* model of dimerization. A single change (P101Q) was introduced in the sequence of a monomeric variant of HP-RNase, in which the N-terminal region and hinge loop were those of the BS-RNase [12]. The substitution, which was carried out in the region where some residues of the RNase A interact to form the open interface of the minor dimer, turned out to be a crucial step toward dimerization. It resulted in the formation of a domain-swapped dimer with a quaternary structure that has hitherto not been described in ribonucleases.

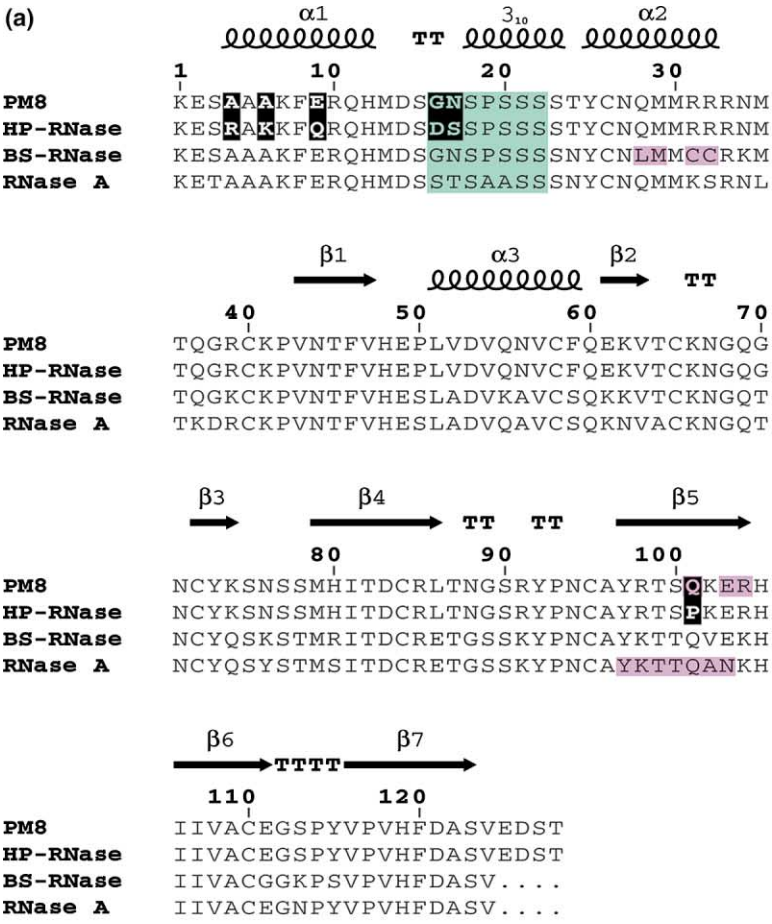
Results

Overall Structure

Human pancreatic ribonuclease PM8 forms dimers with no covalent unions between both chains (A and B). The interchange of entire domains between both subunits is the basis of the dimeric structure. As shown in Figure 2, the N-terminal domain (or tail, T; residues 1–15) of each subunit is nestling against the C-terminal domain (or body, B; residues 23–128) of the other. Such domain swapping generates two composite units (T_A/B_B and T_B/B_A) that can be considered to share their overall structure with the monomeric ribonucleases of their family if the hinge peptide (residues 16–22) is excluded from the comparison. This kind of noncovalent protein association has already been described for the artificial minor dimer of RNase A, although the attained quaternary structure is quite different [5]. Whereas RNase A had to be liophylized in acetic acid to favour dimerization before crystallization, PM8 crystals were grown with no previous treatment of the sample.

The overall size of the dimer is 40 Å $\times$ 40 Å $\times$ 70 Å. Both subunits have the same secondary-structure elements, which are in turn the elements that have been described for a monomeric HP-RNase variant called PM7 [13]. As shown in Figure 3, both chains exhibit three helices (α1, α2, and α3) and seven β strands (β1–β7). Helices α1 and α2 are connected by the hinge peptide, which contains two consecutive turns and therefore forms a 3_{10}-helical structure encompassing residues 18–23. Strand β1 is located between the helices α2 and α3, and the rest of the strands are located, in sequence, after helix α3. Strands β3 and β4 as well as β5 and β6 run anti-parallel and form a twisted β sheet defining the V-shaped cleft where the active site is located. Helices α2 and α3 further delimitate the cleft. Both subunits have four intramolecular disulphide bonds at positions 26–84, 40–95, 58–110, and 65–72. Such bonds are a normal feature in the members of the RNase superfamily. Although when compared to RNase A PM8 has two extra proline residues (positions 19 and 50), the only X-Pro bonds that have a *cis* conformation are those that have one in the bovine enzyme (positions 93 and 114).

Very few differences are detected when both subunits are superimposed (rmsd of 0.7 Å when all the C^α atoms are used). Both domains can be superimposed at once since there is no shift between the tail domains, as was observed in the BS-RNase dimer by Mazzarella et al. [15]. The symmetry operation that relates both subunits is defined by spherical polar angles $\omega = 91.4°$, $\phi = 24.1°$, $\psi = 179.5°$, and a translation of (x, y, z) = (2.1, −5.8,



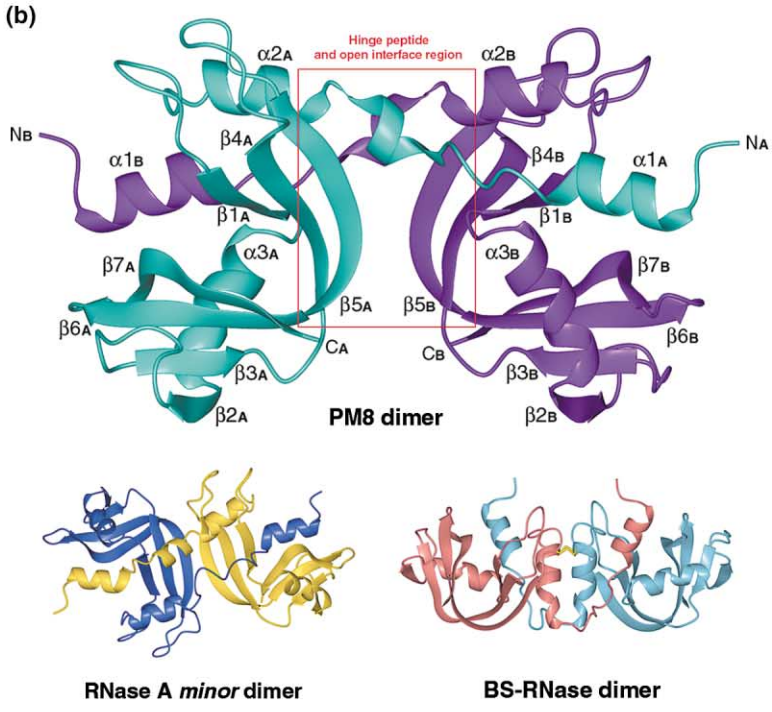


Figure 3. Sequence Alignment and Secondary-Structure Elements of Domain-Swapped Dimeric RNases

(a) Structure-based alignment of the sequences from human pancreatic ribonuclease variant PM8 (present structure), wild-type human pancreatic ribonuclease (HP-RNase), bovine seminal ribonuclease (BS-RNase) and bovine pancreatic ribonuclease (RNase A). Sequence differences between PM8 and HP-RNase are boxed in black. The hinge loop region is boxed in green. Purple boxes show residues involved in the open interface of domain-swapped dimers exchanging their N-terminal α helices. The secondary-structure elements of PM8 (β strands, β; α helices, α; 3_{10}-helices, 3_{10}; and turns, T) are graphically represented and were calculated with the program PROCHECK [35] and DSSP [41]

(b) Ribbon representations of HP-RNase variant PM8, RNase A minor dimer, and BS-RNase dimer. Secondary structure elements of PM8 are labeled according to (a), with helices, strands, and loops shown as helical ribbons, arrows, and thin tubes, respectively.

−18.0) Å [16]. Loop β4β5 (residues 87–96) is disordered in molecule B, and therefore cannot be compared with the same region of molecule A. Deviations larger than 1.0 Å can only be measured around residues 66 and 69 of loop β2β3, with a maximum of 1.98 Å. If one excludes these two loop regions, the rmsd between both subunits is 0.3 Å.

The main difference between both composite active sites is the alternate conformation of the active site His119 in chain B, at the T_A/B_B structural unit. The two conformations, known as *up* and *down*, have been previously found in other RNase A structures [14]. The *down* conformation is the only form found in the structure of the closely related monomeric PM7 [13]. The only significative disagreement between both subunits at the non-catalytic subsites is the position of Lys66, at the p_0 subsite. In fact, the divergence arises from the different conformation of the whole loop β2β3 in chains A and B. The average temperature factor is 26.0 $Å^2$ for chain A and 25.2 $Å^2$ for chain B.

Hinge Peptides

Both hinge peptides (residues 16–22) have the same conformation. This is a unique feature in known dimeric ribonucleases with intertwined N-terminal α helices. Although the model used for the molecular replacement did not include the atomic coordinates for residues 18–21, the structure of both hinge peptides clearly showed up in the earliest refinement cycles of PM8. The low temperature factors found for residues 19 and 20 (around 26 $Å^2$) confirm that it is a well-ordered region. Although our dimer shares the primary sequence of the hinge peptide with BS-RNase, it has a different structure. There are two contiguous turns in the middle of the linker peptides between residues 18 and 23, so that the hinge loop in fact adopts a 3_{10} helix conformation. Both turns are stabilized by multiple-centred H bonds (Figure 4). The helical structure is capped by H bonds between O^γ atoms of serine residues 21 and 22, and the amide nitrogen atoms of Ser18 and Ser21, respectively. Several kinds of turns have been described in hinge loops of different ribonucleases [17], but a clear helical structure has only been found in one of the linking peptides of artificial RNase A domain-swapped minor dimer.

The presence in PM8 of a Pro residue at position 19, together with a Gly at position 16, provides the minimum requirements for a natural swapping of N-terminal regions. The hinge peptides of BS-RNase and HP-RNase differ only in two residues (positions 16 and 17; see Figure 2a). Both proteins have a Pro residue at position 19, which is necessary for a natural swapping [18], but BS-RNase has a distinctive Gly at position 16 that is likely to provide the necessary degree of freedom for the change of conformation. In fact, the position of Gly16B is uncertain in PM8 due to its mobility.

Open Interface

There are no significant differences among the closed interfaces of the N-terminally swapped RNase dimers that have been published to date [5, 6, 15]. Thus, variations in the overall quaternary structure are a consequence of the interactions taking place along the open interface. The substitution of the Pro101 for a Gln in PM8 enhances the stability of the hinge peptide and also takes part in the formation of the open interface. Clear hydrogen bonds are found between atoms of both chains as a result of the substitution. Each Gln101 residue establishes three H bonds, one between its amide N atom and the O^γ atom of Ser20 of its own chain, the other two between its $O^{\epsilon 1}$ atom and the N and O^γ atoms of Ser20 of the other subunit (Figures 4b and 5). Moreover, Gln101 participates in a stacking interaction that involves different residues of both chains. The two Pro19 residues of the dimer are stacked between the side chains of residues Gln101 and Tyr25 of the other chain (Figure 4b). The interactions between subunits are therefore stronger, and the stability of the hinge peptide is improved by four H bonds that could not be established if residue 101 were a proline.

The interactions described above play the role of a secondary anchor point, after the formation of the closed interface between T and B domains, and determine the relative orientation of both subunits to some extent. The final quaternary structure is attained when, as a result of this particular orientation, two antiparallel ionic interactions take place between residues of different chains. The Glu103 residues of each subunit establish a salt bridge with the Arg104 residues of the other (Figure 5). The distances between atoms OE2 and NH1 of the ionic pairs are 2.9 Å for Glu103A-Arg104B and 3.0 Å for Arg104A-Glu103B. In fact, both residues are located in the same β strand (β5) that is involved in the formation of the open interface of the minor RNase A domain-swapped dimer [5], although the interactions are of a different nature. These are the only direct contacts between the two subunits—apart from those that form the closed interface and the interactions at the hinge peptide—although several water-mediated hydrogen bonds are also observed along the open interface.

Nondenaturing Cathodic Electrophoresis and RI Binding Assays

PM8 dimers are not restricted to the crystal state. Nondenaturing cathodic electrophoresis reveals that oligomeric forms of PM8 may be found when the protein is in solution (Figure 6). Although the monomer is quantitatively the major form, at least two more bands appear when the amount of protein loaded onto the gel is large enough (10 μg). The major form is assigned to the PM8 monomer as it comigrates with the monomeric control (RNase A). The other two forms have a slower electrophoretic mobility than the monomer and may be assigned to putative PM8 oligomers. The structure described in the present work may correspond to one of these slowly moving forms of PM8.

Cytotoxicity is one of the biological actions acquired by BS-RNase upon dimerization [4]. The cytotoxic effects have been related to the ability of BS-RNase to evade the proteic ribonuclease inhibitor (RI) [19] as a result of its quaternary structure. The interaction of RI with the PM8 HP-RNase variant has been tested in an agarose gel-based assay. Under assay conditions, PM8 is inhibited by RI to the same extent as RNase A and PM7 [13]. However, the PM8 concentration in the assay

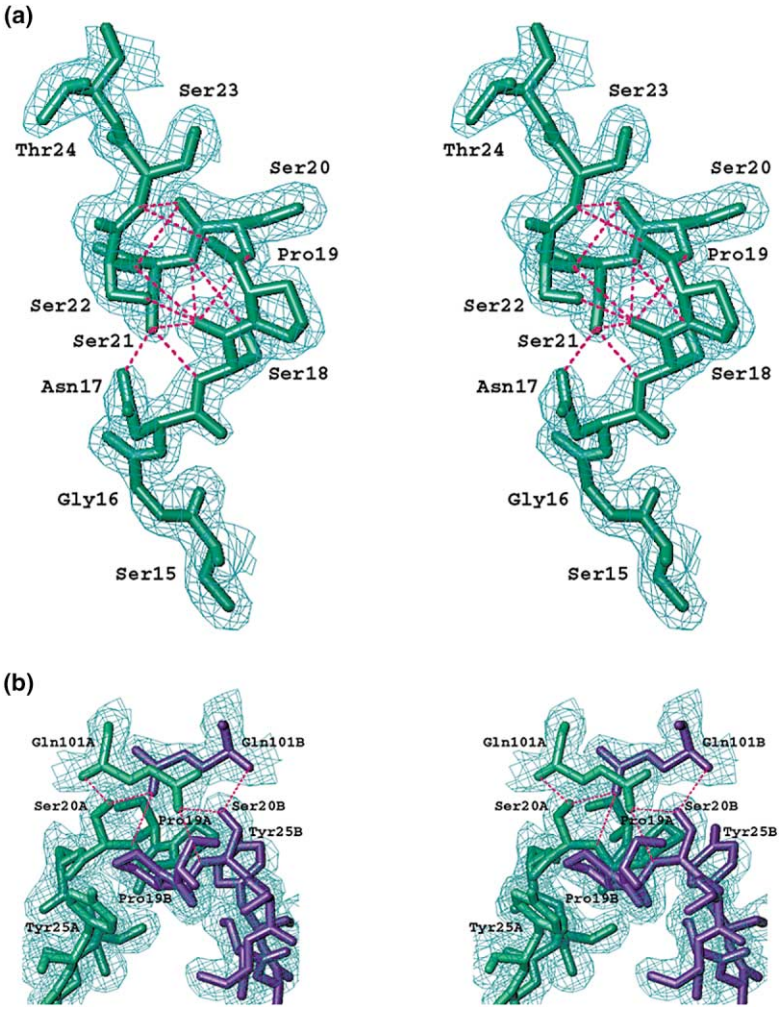


Figure 4. Structure of the Hinge Peptide and Interaction with Gln101

Stereo views of (a) the hinge peptide in chain A forming a 3_{10} helix structure and (b) the H bonds and stacking interactions between Gln101 and residues Pro19 and Ser20 (see text). The final σA $2F_o - F_c$ electron density maps (contoured at 1.0 σ) and the refined model are presented in both images. Red dashed lines indicate hydrogen bonds. Chains A and B are colored green and purple, respectively.

is very low, and thus the oligomeric population may be enzymatically undetectable.

Discussion

From a Preevolved Hybrid to a Hybrid Dimer

PM8 is a single-residue variant of an engineered protein called PM5, which has a hybrid amino acid sequence between BS-RNase (tail domain and hinge peptide) and HP-RNase (body domain) [12]. PM5 may be considered a preevolved protein since it has the appropriate hinge peptide sequence for natural domain swapping [18], in addition to a tail domain that may interact with the body domain of a neighboring molecule. Because an N-terminal exchange is favored in PM5, one might expect the formation of either a dimer resembling the M $\times$ M BS-RNase form or the RNase A minor dimer. A BS-RNase-like dimer cannot be achieved due to the lack of cysteines 31 and 32, which form two intersubunit disulfide bonds in the seminal enzyme. A structure such as that of the RNase A minor dimer is probably difficult to attain as well since the interactions along two neighboring β5 strands would be of a different nature as a result of the aminoacid sequence of PM5 (see Figure 3a). The substitution of a Pro at position 101 by a Gln (as in strand β5 of RNase A) in PM8 enables new interactions at a new open interface to promote the formation of a never-reported dimeric structure, half way between the natural BS-RNase dimer [15] and the artificial RNase A minor dimer [5].

Apart from the obvious similarity in the closed interfaces, dimeric PM8 has features in common with both domain-swapped RNase dimers. These features arise from the swapping and quaternary-structure determinants that PM8 shares with them. As in the BS-RNase dimer, the exchange of N-terminal domains occurs in PM8 on opposite sides of the dimeric structure (see Figure 1). This kind of exchange is probably favored by the rigid structure of the hinge peptide, which has an identical amino acid sequence in both dimers. In the RNase A minor dimer the exchange of N-terminal do-

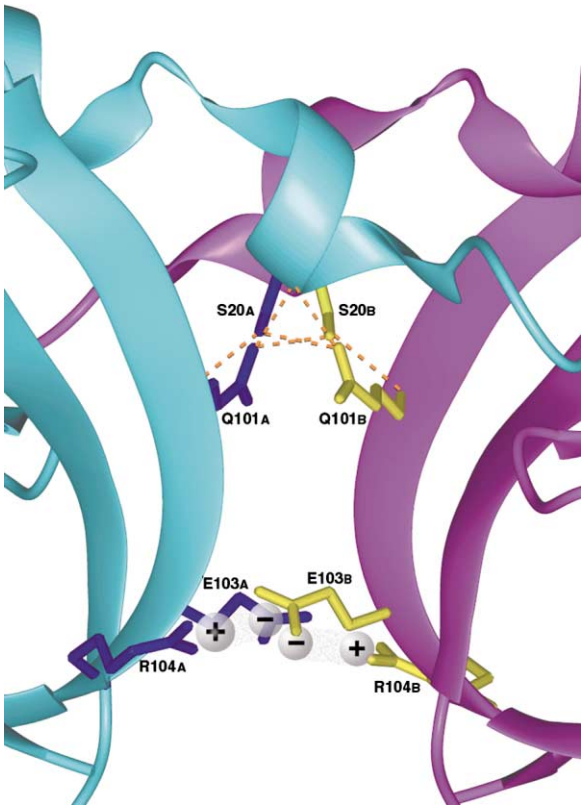


Figure 5. Interactions Along the Open Interface

Detail of the region squared in Figure 2b shows a schematic representation of the interactions that take place in the open interface. Red dashed lines indicate intermolecular and intramolecular hydrogen bonds between Gln101 residues and Ser20 residues of both chains. Antiparallel ionic pairs (residues Glu103 and Arg104 of both chains) are depicted with white spheres labeled with their corresponding charge sign.

mains takes place along the same side of the quaternary structure. As a result of the residue substitution (P101Q), the new open interface formed in PM8 resembles that of the RNase A dimer. Adjoining residues are in both cases located in strand β5 (residues 97–104; see Figure 3a), although the nature of the interactions that maintain the quaternary conformation is rather different. In BS-RNase, the open interface is formed primarily between two α helices (residues 24–33).

Diverse Open Interfaces Are Attainable

As Eisenberg et al. proposed in their description of the RNase A domain-swapped minor dimer [5], mutations can influence the nature of the open interface and hence the overall structure of ribonuclease dimers. The dimeric structure of PM8 confirms this idea since a Gln at position 101 influences the nature of the open interface. This single substitution affects the conformation of the hinge peptide as well by promoting the formation of a 3_{10} helix structure. This helical structure shortens the distance between the tail and body domains. Hinge loop shortening is a common strategy for obtaining domain-swapped dimers [20, 21], and Russo et al. [22] obtained a stable HP-RNase dimer by deleting 3–5 residues from the hinge peptide. Their dimer was assumed to have a similar structure to that of the RNase A dimer. However, as shown in the present work, other quaternary conformations can be attained, which could be the case for Russo's dimer as well.

There are at least two conformations of dimeric RNase A, and they can be separated by nondenaturing cathodic electrophoresis, size-exclusion chromatography [23], or ion-exchange chromatography [24]. Eisenberg et al. have reported the three-dimensional structure of both RNase A dimer forms [5, 6]. They have shown that the minor dimer form is based on the exchange of the N-terminal α helices, whereas the C-terminal β strands are the exchanged elements in the major form. Thus, it is possible to obtain more than one open interface that has a different quaternary structure even when no substitutions are made. Recently, Park and Raines have detected a recombinant RNase A dimer at neutral pH, which suggests that RNase A has an intrinsic ability to form a functional domain-swapped dimer even in physiological conditions [25]. The three-dimensional structure of this dimer remains unknown.

Dimers in Solution, RNase Activity, and Resistance to RI

Although most PM8 molecules exist in the monomeric form when they are in solution at different pH and protein concentration values, the presence of a few dimeric or oligomeric forms is also confirmed. The interactions found along the open interface of our dimer are too weak to ensure an important population of dimeric HP-RNase in solution. Ionic strength may affect the stability of the dimer since the union is partially based on two electrostatic interactions. These interactions could be favored by the low dielectric constant of the precipitant solution.

Both composite active sites of dimeric PM8 are potentially functional, as the architecture of each is identical to that of its monomeric relative, PM7 [13]. However, the ability of the dimer to cleave RNA cannot be confirmed. The monomeric population of the protein, which is quantitatively more important and difficult to separate, hampers an accurate analysis of its catalytic properties.

Unlike dimeric BS-RNase, our variant is RI sensitive, as shown by binding assays. This means that PM8 is not able to degrade RNA when the ribonuclease inhibitor is present. The PM8 concentration is very low in the interaction assay, and under this condition the oligomeric forms could be undetectable. RI binding of RNases is mainly located at loops α2β1, β2β3, and β4β5 of the enzyme [26, 27], and the quaternary structure of BS-RNase may prevent the union since access to loops α2β1 and β4β5 is blocked. Our dimeric structure is unlikely to evade the RI because access to these loops is possible, at least for one RI molecule. The binding of a second RI molecule to ensure the blocking of the second active site of PM8 is not possible due to steric hindrance, unless the overall quaternary conformation of the dimer is altered. However, it is possible that even an initial complex with one RI molecule alters the structure in such a way that both composite active sites become nonfunctional or, alternatively, the dimer is dissociated and the equilibrium is displaced toward the monomeric form.

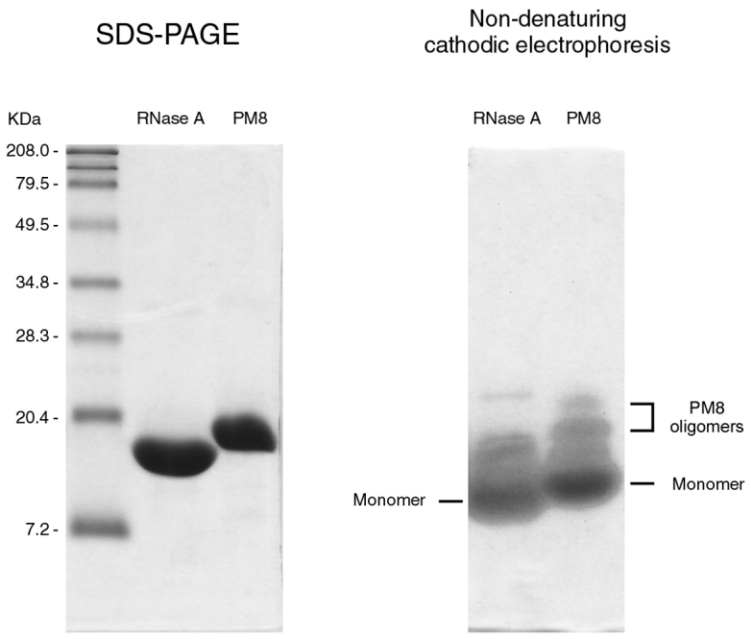


Figure 6. Denaturing and Nondenaturing Cathodic Electrophoresis Gels of Protein PM8 in Solution

Only one band appears when 10 μg of protein PM8 at 10 mg/ml is loaded onto a SDS-PAGE gel. At least three bands appear when the same amount of protein is loaded onto a nondenaturing cathodic electrophoresis gel, although the major part of the sample can be assigned to the monomeric form by comparison of its electrophoretic mobility with that of control RNAse A (four residues shorter than PM8, and with a slight tendency to dimerize as can be seen on the gel). Slowly moving bands can therefore be assigned to oligomeric associations of PM8.

Molecular Evolution

The domain swapping in PM8 provides clues on how to promote protein association and widen the availability of new and different quaternary conformations that may be of biological interest. As in the *swap early* model for the evolution of intertwined dimers [1], domain interchange in PM8 is an event that has been fixed with a single amino acid substitution (P101Q). This single mutation fastens the swapping in PM8 (hydrogen bonds and stacking interactions with residues in the hinge loop) while promoting the formation of a new open interface nearby (antiparallel ionic pairs between residues 103 and 104). Accordingly, an almost simultaneous enhancement of swapping and covalent union formation is assumed in a model for the pathway from a monomeric to a dimeric form of BS-RNase [28], which is consistent with the reconstructed evolutionary history of the seminal RNase gene family. Our dimer is also consistent with the model proposed by Park and Raines [25], which implies a continuous evolution performed on an unstable form in physiological conditions until the formation of a more stable dimer is reached.

Hence, a *swap early* model is better suited for the in vitro evolution of our dimer. An additional mutation fastening the now rather fragile open interface of PM8 would probably produce a more abundant dimeric form in solution and perhaps a different quaternary structure endowed with new biological activities.

Biological Implications

To gain a deeper insight into molecular evolution, one must understand the structural basis of protein oligomerization. The protein association known as "domain swapping" offers a fast way to evolve from a monomer to a stable oligomer with new biological actions. Because they are based on the interchange of complete secondary elements between identical molecules, the interactions that take place between intertwined regions are guaranteed and can play the role of a primary anchor point. From then on, very few mutations will be needed to reinforce the union with a new interface between subunits.

The crystal structure of RNase PM8 proves that a new

Table 1. Data Collection and Refinement Statistics

Data Collection	
Resolution range (Å)	13.0–2.0
Completeness (%)	99.4 (98.8)
Number of observed reflections	56,729
Number of unique reflections	16,247
R_{merge}[1] (%)	7.6 (31.5)
$\langle I \rangle / \langle \sigma I \rangle$	7.1 (2.4)
Multiplicity	3.4 (3.1)
Refinement	
Number of reflections in working set	14,635
Number of reflections in test set	1,610
Number of protein atoms in the asym. unit	1,987
Number of solvent molecules in the asym. unit	342
R^2, R_{free}[3] (%)	19.6, 24.3
Rms deviations from target values	
Bond lengths (Å)	0.006
Bond angles (°)	1.565
Average B factors (Å^2)	
Main chain	25.6
Side chain	27.1
Water molecules	42.0
All atoms	28.7
Wilson plot	25.4
Ramachandran statistics (%)	
Most-favored regions	88.2
Additionally allowed regions	11.8
Generously allowed regions	0.0
Disallowed regions	0.0

[1] $R_{merge} = \Sigma|I_h - \langle I_h \rangle| / \Sigma \langle I_h \rangle$, where $\langle I_h \rangle$ is the average of intensity of reflection h and their symmetry related reflections.
[2] $R = \Sigma||F_o| - |F_c|| / \Sigma|F_o|$.
[3] R_{free} = R–value calculated for 10% of reflections not used for the refinement. The outermost resolution shell (2.12–2.00 Å) values are shown in parentheses.

dimeric structure can be attained by only one residue change. The substitution of residue Pro101 by a glutamine enables the formation of four additional hydrogen bonds and a stacking interaction that strengthens the domain-swapped structure at the hinge peptide and determines the relative position of both subunits. As a result of this particular orientation, two intermolecular salt bridges that fasten the quaternary structure are established.

Although other domain-swapped dimers have been described in the ribonuclease superfamily, the overall structure of dimeric PM8 is new. The achievement of this swapped dimer validates a strategy for rapidly changing protein architecture in vitro and suggests that similar processes might have occurred during evolution, resulting in many of the present oligomeric proteins.

Experimental Procedures

Sample Preparation

Proteins PM5 and PM8 come from the expression of a hybrid gene in *Escherichia coli* BL21(DE3). The gene encoding PM5 was constructed with parts of the genes encoding BS-RNase (residues 1–20) and HP-RNase (residues 21–128). Protein PM8 is a variant of PM5 that we obtained with site-directed mutagenesis to introduce the P101Q change. Such constructs as well as the expression and purification of both proteins are described in detail elsewhere [12].

Crystallization and Data Collection

Crystals of PM8 grew by the vapor diffusion technique at room temperature from hanging drops containing 2 μl of 10 mg/ml protein and 2 μl of precipitant solution containing 20% (v/v) ethanol and 0.1 M bicine pH 9.0, equilibrated against 1 ml of precipitant solution. The crystals had a rod shape of approximately $0.8 \times 0.2 \times 0.2$ mm^3 and grew in clusters. One of the branches of a cluster was used for data collection.

X-ray data were collected "in house" with a Mar Research image plate (30 cm), mounted on a Rigaku RU 200 rotating anode generator providing graphite monochromed CuK_α radiation. The crystals were frozen at 110 K under a N_2 stream. In order to cryoprotect the crystals, we added 20% of MPD (2-methyl-2,4-pentanediol) to the mother liquor.

The crystals diffracted up to 2.0 Å and belong to space group $P2_12_12_1$ with unit cell dimensions of a = 50.61 Å, b = 61.42 Å, and c = 75.14 Å. Data were indexed, processed, and scaled with MOSFLM [29] and SCALA [30]. Data collection statistics are shown in Table 1.

Structure Determination and Refinement

The Matthews coefficient (V_m = 2.13) suggested two molecules of monomeric RNase per asymmetric unit. The structure was solved by the molecular replacement method with AMoRe [31]. A monomeric homolog PM7 (Protein Data Bank code 1dza), which only differs by two residues from PM8, was used as a search model. The correct solution of the molecular replacement, calculated at 3.5 Å, had a correlation coefficient of 52.1% and an R factor of 40.5% (highest noise peak at 24.3% and 50.6%, respectively). The absence of the hinge loop in the refined structure of PM7 (residues 17–22) left the entire swapping zone free of bias. The hinge loop was clearly defined in the σA $2F_o - F_c$ and $F_o - F_c$ electron density maps, and its building was straightforward, even before the start of refinement.

Refinement of the structure with CNS [32] began with a cycle of rigid body refinement and then a simulated annealing to reduce bias from the model. This helped to define some zones of the most mobile loops. The initial steps of positional refinement were made at a resolution of 2.3 Å with noncrystollographic-symmetry restraints that were taken out once the refinement converged. Using TURBO [33], we alternated cycles of positional and temperature refinement with manual building. All the data were used during refinement, without sigma or resolution cut-offs. Bulk solvent correction was applied. We added water molecules by using the automatic protocol of ARP-WARP [34] and visually checked their position once the R factors dropped below 28%. In the final cycles of refinement, alternated side chain conformations were built for residues 32, 75, and 91 of molecule A and for residues 78, 100, and 119 of molecule B.

The final model contains 342 molecules of water and has an R factor of 0.196 and an R_{free} of 0.243. Refinement statistics are shown in Table 1. The structure has excellent geometry, as was validated with PROCHECK [35]. B factor statistics were calculated with BAVERAGE from the CCP4 package [36]. Least squares superpositions were performed with TURBO. Figures were drawn with TURBO and MOLMOL [37].

Detection of Dimeric Forms in Solution and Ribonuclease Inhibitor Binding Assay

Pure samples of PM8 and monomeric RNase A (as a control) were analyzed by cathodic gel electrophoresis under nondenaturing conditions consisting of a β-alanine/acetic acid buffer (pH 4.0), according to the methods of Reisfeld et al. [38]. Polyacrylamide (7.5%) was used, and gels were run at 20 mA for 1 hr at 4°C. An amount of 10 μg of protein at a 10 mg/ml concentration was loaded onto a nondenaturing gel. Loading the same amount of protein onto SDS-PAGE gels [39] confirmed the purity of the sample.

The ribonucleolytic activity of PM8 in the presence of porcine RI was tested with an agarose gel-based assay according to the methods of Wu et al. [40] as described by Pous et al. [13].

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References

1. Bennett, M.J., Choe, S., and Eisenberg, D.S. (1994). Domain swapping: entangling alliances between proteins. Proc. Natl. Acad. Sci. USA. *91*, 3127–3131.
2. Raines, R.T. (1998). Ribonuclease A. Chem. Rev. *98*, 1045–1065.
3. D'Alessio, G., Di Donato, A., Mazzarella, L., and Piccoli, R. (1997). Seminal ribonuclease: the importance of diversity. In Ribonucleases: Structures and Functions, G. D'Alessio and J.F. Riordan, eds. New York: Academic Press), pp. 383-423.
4. Crestfield, A.M., Stein, W.H., and Moore, S. (1962). On the aggregation of bovine pancreatic ribonuclease. Arch. Biochem. Biophys. Suppl *1*, 217–222.
5. Liu, Y., Hart, P.J., Schlunegger, M.P., and Eisenberg, D. (1998). The crystal structure of a 3D domain-swapped dimer of RNase A at 2.1-Å resolution. Proc. Natl. Acad. Sci. USA *95*, 3437–3442.
6. Liu, Y., Gotte, G., Libonati, M., and Eisenberg, D. (2001). A domain-swapped RNase A dimer with implications for amyloid formation. Nat. Struct. Biol. *8*, 211–214.
7. D'Alessio, G. (1999). Evolution of oligomeric proteins. The unusual case of a dimeric ribonuclease. Eur. J. Biochem. *266*, 699–708.
8. Bennett, M.J., Schlunegger, M.P., and Eisenberg, D.S. (1995). 3D domain swapping: a mechanism for oligomer assembly. Protein Sci. *4*, 2455–2468.
9. Di Donato, A., Cafaro, V., and D'Alessio, G. (1994). Ribonuclease A can be transformed into a dimeric ribonuclease with antitumor activity. J. Biol. Chem. *269*, 17394–17396.
10. Piccoli, R., et al., and D'Alessio, G. (1999). A dimeric mutant of human pancreatic ribonuclease with selective cytotoxicity toward malignant cells. Proc. Natl. Acad. Sci. USA *96*, 7768–7773.

11. Beintema, J.J., Wietzes, P., Weickman, J.L., and Glitz, D.G. (1984). The amino acid sequence of human pancreatic ribonuclease. Anal. Biochem. *136*, 48–64.
12. Canals, A., Ribó, M., Benito, A., Bosch, M., Mombelli, E., and Vilanova, M. (1999). Production of engineered human pancreatic ribonucleases, solving expression and purification problems, and enhancing thermostability. Protein Expr. Purif. *17*, 169–181.
13. Pous, J., et al., and Coll, M. (2000). Three-dimensional structure of a human pancreatic ribonuclease variant, a step forward in the design of cytotoxic ribonucleases. J. Mol. Biol. *303*, 49–59.
14. Borkakoti, N., Moss, D.S., and Palmer, R.A. (1982). Ribonuclease-A: least-squares refinement of the structure at 1.45 Å resolution. Acta Crystallogr. B *38*, 2210–2217.
15. Mazzarella, L., Capasso, S., Demasi, D., Di'Lorenzo, G., Mattia, C.A., and Zagari, A. (1993). Bovine seminal ribonuclease-structure at 1.9 Å resolution. Acta Crystallogr. D *49*, 389–402.
16. Kabsch, W. (1976). A solution for the best rotation to relate two sets of vectors. Acta. Crystallogr. A *32*, 922–923.
17. Vitagliano, L., Adinolfi, S., Riccio, A., Sica, F., Zagari, A., and Mazzarella, L. (1998). Binding of a substrate analog to a domain swapping protein: X-ray structure of the complex of bovine seminal ribonuclease with uridylyl (2′,5′)adenosine. Protein Sci. *7*, 1691–1699.
18. Mazzarella, L., Vitagliano, L., and Zagari, A. (1995). Swapping structural determinants of ribonucleases: an energetic analysis of the hinge peptide 16-22. Proc. Natl. Acad. Sci. USA. *92*, 3799–3803.
19. Murthy, B.S., and Sirdeshmukh, R. (1992). Sensitivity of monomeric and dimeric forms of bovine seminal ribonuclease to human placental ribonuclease inhibitor. Biochem. J. *281*, 343–348.
20. Green, S.M., Gittis, A.G., Meeker, A.K., and Lattman, E.E. (1995). One-step evolution of a dimer from a monomeric protein. Nat. Struct. Biol. *2*, 746–751.
21. Ogihara, N.L., Ghirlanda, G., Bryson, J.W., Gingery, M., DeGrado, W.F., and Eisenberg, D. (2001). Design of three-dimensional domain-swapped dimers and fibrous oligomers. Proc. Natl. Acad. Sci. USA *98*, 1404–1409.
22. Russo, N., Antignani, A., and D'Alessio, G. (2000). In vitro evolution of a dimeric variant of human pancreatic ribonuclease. Biochemistry *39*, 3585–3591.
23. Sorrentino, S., et al., and D'Alessio, G. (2000). The two dimeric forms of RNase A. FEBS Lett. *466*, 35–39.
24. Gotte, G., and Libonati, M. (1998). Two different forms of aggregated dimers of ribonuclease A. Biochim. Biophys. Acta *1386*, 106–112.
25. Park, C., and Raines, R.T. (2000). Dimer formation by a "monomeric" protein. Protein Sci. *9*, 2026–2033.
26. Papageorgiou, A.C., Shapiro, R., and Acharya, K.R. (1997). Molecular recognition of human angiogenin by placental ribonuclease inhibitor—an X-ray crystallographic study at 2.0 Å resolution. EMBO J. *16*, 5162–5177.
27. Kobe, B., and Deisenhofer, J. (1996). Mechanism of ribonuclease inhibition by ribonuclease inhibitor protein based on the crystal structure of its complex with ribonuclease A. J. Mol. Biol. *264*, 1028–1043.
28. Ciglic, M.I., et al., and Benner, S.A. (1998). Origin of Dimeric Structure in the Ribonuclease Superfamily. Biochemistry *37*, 4008–4022.
29. Leslie, A. (1991). Macromolecular data processing. In Crystallographic Computing V, D. Moras, A.D. Podjarni, and J.C. Thierry, eds. Oxford, UK: Oxford University Press, pp. 27-38.
30. Evans, P.R. (1993). Data reduction. In Proceedings of CCP4 Study Weekend, 1993, on Data Collection and Processing, compiled by L. Sawyer, N. Isaac, and S. Bailey. (Daresbury, UK), pp. 114-122.
31. Navaza, J. (1994). AMoRe: an automated package for molecular replacement. Acta Crystallogr. A *50*, 157–163.
32. Brünger, A.T., et al., and Warren, G.L. (1988). Crystallography and NMR system: a new software suite for macromolecular structure determination. Acta Crystallogr. D *54*, 905–921.
33. Roussell, A., and Cambilleau, C. (1989). TURBO-FRODO. Mountain View, CA: Silicon Graphics, pp. 77-79.
34. Perrakis, A., Sixma, T.K., Wilson, K.S., and Lamzin, V.S. (1997). wARP: improvement and extension of crystallographic phases by weighted averaging of multiple refined dummy atomic models. Acta Crystallog. D *53*, 448–455.
35. Laskowski, R.A., MacArthur, M.W., Moss, D.S., and Thornton, J.M. (1993). PROCHECK: a program to check the stereochemical quality of protein structures. J. Appl. Crystallogr. *26*, 283–291.
36. CCP4 (Collaborative Computational Project 4) (1994). The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D *50*, 760-763.
37. Koradi, R., Billeter, M., and Wuthrich, K. (1996). MOLMOL, a program for displaying and analysis of macromolecular structures. J. Mol. Graph. *14*, 51–55.
38. Reisfeld, R.A., Lewis, U.J., and Williams, D.E. (1962). Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature *195*, 281–283.
39. Ausubel, F.M., et al., and Struhl, K. (1989). Current Protocols in Molecular Biology. New York: Wiley.
40. Wu, Y., Mikulski, S.M., Ardelt, W., Rybak, S.M., and Youle, R.J. (1993). A cytotoxic ribonuclease: study of the mechanism of onconase cytotoxicity. J. Biol. Chem. *268*, 10686–10693.
41. Kabsch, W., and Sander, C. (1983). Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. Biopolymers *22*, 2577–2637.

Accession Numbers

Atomic coordinates have been deposited with the Brookhaven Protein Data Bank (http://www.pdb.org) under accession code 1h8x.