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A Hinge Region *Cis*-proline in Ribonuclease A Acts as a Conformational Gatekeeper for C-terminal Domain Swapping

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Abstract

Domain swapping, the process in which a structural unit is exchanged between monomers to create a dimer containing two versions of the monomeric fold, is believed to be an important mechanism for oligomerization and the formation of amyloid fibrils. Structural studies have implicated proline as an important residue for domain swapping due to its increased frequency in hinge regions preceding swapped arms. We hypothesized that prolines unique ability to populate both *cis* and *trans* peptide bond conformations may allow proline to act as a conformational gatekeeper, regulating interconversion between monomer and domain-swapped dimer forms. The hinge region of RNase A contains a proline at residue 114 that adopts a *cis* conformation in the monomer and extends to a *trans* conformation in the C-terminal domain-swapped dimer. Substitution of P114 with residues that strongly prefer a *trans* peptide bond (Ala, Gly) results in significant population of the C-terminal domain-swapped dimer under near-physiological conditions (pH 8.0, 37°C). This is in stark contrast to dimerization of wild-type RNase A, which requires incubation under extreme conditions such as lyophilization from acetic acid or elevated temperature. In addition, we observe similar results when *cis*-P114 is mutated to glycine in a homologous RNase, human pancreatic RNase 1. Our results suggest isomerization at P114 may facilitate population of a partially unfolded intermediate or alternative structure competent for domain swapping, and provide support for a more general role for proline isomerization as a conformational gatekeeper in domain swapping and oligomerization.

Keywords

Domain swapping; oligomerization; proline isomerization; ribonuclease A; protein folding

Three dimensional domain swapping is a mechanism for protein oligomerization in which monomers exchange identical structural units resulting in a quaternary structure with subunits that recapitulate the monomeric fold. This process has been implicated across a range of functions, from evolution of quaternary structure to enzyme regulation, with a particular emphasis on its potential role in protein aggregation and amyloid formation¹.

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Domain swapping and amyloid formation share common features such as self-complementarity and the potential to retain native structure and function in the context of a larger oligomeric assembly.

In 2005, Eisenberg and co-workers demonstrated that an engineered version of bovine pancreatic RNase A, a classic model system for studying protein folding and domain swapping, was capable of forming amyloid fibrils². Importantly, these fibrils showed enzymatic activity that could only be attributed to the presence of native folds formed via domain swapping. These studies therefore provide a direct link between domain swapping and amyloid formation, and bring new significance to using RNase A as a model system for investigating the mechanism of domain swapping.

Wild-type RNase A, when lyophilized from 50% acetic acid, will domain swap and form either an N-terminal or C-terminal swapped dimer. In the N-terminal dimer, the first helix is swapped between each monomeric unit³, whereas in the C-terminal dimer, a β -strand consisting of residues 116–124 is exchanged⁴. The swapped C-terminal β -strand is anchored to the rest of the protein by a hinge region (residues 112–115) which undergoes a conformational change upon domain swapping, forming the only unique contacts in the dimer interface, also known as the open interface⁴ (Figure 1). The hinge region from each monomer extends to form β -strands that align at the open interface and form β -stacking interactions reminiscent of the polar zipper characteristic of amyloid fibrils⁵. Sambashivan et al.² inserted a poly-glutamine sequence into this hinge region to create a version of RNase A that domain swaps and forms amyloid fibrils. Understanding the mechanism for the C-terminal dimerization of RNase A is, therefore, an important step towards understanding the link between domain swapping and amyloid formation.

Oligomeric forms of RNase A have also generated interest as engineered therapeutics, as they have been shown to have important anti-tumor properties^{6, 7}. However, it is difficult to induce high yields of dimer from WT RNase A, and once the dimers are formed they appear to be metastable, eventually reverting back to monomers. Thus, there has been significant interest in engineering dimers and higher order oligomers of RNase A that form more readily and persist in their dimeric form, usually via some sort of covalent cross-linkage^{6, 8}. Gaining a better understanding of the mechanism by which these dimers form is important not only for understanding the process of domain swapping but also for improving methods by which to engineer stable dimeric forms.

There is very little structural or energetic data to explain the mechanism that leads to the C-terminal swapped dimer. One suggestion has been that dimerization occurs from the unfolded state, although dimer is not observed when RNase A is refolded from standard denaturing conditions⁴; however, under dimer-inducing conditions, more denaturing conditions favor the formation of the C-terminal dimer over the N-terminal dimer⁹. An alternative hypothesis is that dimerization proceeds via a local unfolding event¹⁰.

There are some data that suggest the existence of a partially unfolded species with an exposed C-terminal β -strand that could serve as an exchange-competent species. Studies in the early 1970's revealed that RNase A variants lacking the last several residues of the C-terminal β -strand are not globally unfolded^{11, 12}. These truncated RNases A show spectroscopic signals of residual structure but lack significant thermodynamic stability, suggesting that removal of this strand does not lead to global unfolding of the protein, but results in a high energy partially-folded state that may serve as an intermediate en route to dimerization.

While the pathway leading to formation of the C-terminal dimer is poorly understood, many studies have shown that dimerization of RNase A can be initiated by subjecting the protein

to a variety of non-physiological conditions such as lyophilization from 50% acetic acid, exposure to non-aqueous solvents, or elevated temperatures⁹. These studies suggest a barrier exists to forming the dimer that can be overcome under extreme conditions. In addition, there is no obvious reason why the C-terminal dimer should be thermodynamically stable compared to the native monomer. The enthalpic interactions gained at the open interface are relatively minimal and, at low protein concentration, are unlikely to balance the entropic cost of dimerization. There must be some subtle conformational changes that allow for dimer formation.

The one major difference between the crystal structures of the RNase A monomer and the C-terminal domain-swapped dimer is in the four-residue hinge region: proline 114 is *cis* in the monomer and *trans* in the C-terminal domain-swapped dimer⁴ (Figure 1). Sequence gazing has suggested that proline may play an important role in domain swapping; Bergdoll et al. 13 conducted a survey of oligomeric structures in the protein data bank that contained domain-swapped elements and found an increased frequency of prolines at the base of the exchanged arms. Experiments on p13suc1 have supported this hypothesis, suggesting that the unique conformational rigidity of proline may play an important role in imparting or relieving the backbone strain involved in domain swapping^{14, 15}. The unique ability for X-Pro bonds to populate both the *cis* and *trans* conformations, which has been shown to affect many cellular and molecular processes, may also affect domain swapping. Interestingly, proline isomerization is known to play a role in the mechanism of oligomerization and amyloid formation for some proteins that involve interactions similar to domain swapping, including β 2-microglobulin dimerization and stefin B tetramer formation^{16, 17, 18}.

We hypothesized that proline isomerization acts as a conformational gatekeeper in the conversion of protein monomers to domain-swapped dimers, and that the presence of proline in the hinge region of RNase A may have a significant effect on its propensity for domain swapping. We used site-directed mutagenesis to create RNases A that will preferentially populate either a *cis* or *trans* conformation at residue 114. Indeed, the crystal structure of P114G indicates that the peptide backbone now adopts a *trans* conformation (Fig 1)¹⁹. If proline isomerization plays an important role in domain swapping, then we expect a significant difference in the swapping behavior of RNase A variants with *cis* and *trans* at the peptide bond between residues 113 and 114, and this effect should be generalizable to other homologous RNases.

Results

To investigate the role of P114 in the domain swapping of bovine pancreatic ribonuclease A (BP-RNase A), we studied two different variants at this position: P114G and P114A. While P114 is *cis* in the native structure, the P114G and P114A variants are believed to adopt a *trans* conformation^{19, 20}. Both of these variants were made in the background of the active-site mutation H119A and the three proteins are referred to as WT*, P114G*, and P114A*. BP-RNase A contains two *cis* X-proline bonds, position 114 in the C-terminal hinge region and position 93 in the interior of the protein. To control for changes in global stability and any proline-specific effects not localized to the hinge region, we also constructed the variant P93A in the background of H119A (P93A*).

Effects on Protein Stability

Purified variants of BP-RNase A were evaluated for folding and stability using standard circular dichroism (CD) spectroscopy. The free energy of unfolding (ΔG_{unf}) was determined by chemical-induced denaturation and fit using a two-state linear extrapolation²¹ (Figure 2). All three variants are less stable than WT* by ~2 kcal/mol (Table 1).

Dimerization propensity

To measure the relative swapping propensities of the BP-RNase A mutants, the proteins were first incubated at 10 mg/mL in 100 mM Tris, pH 8.0 at 37°C for 24 hours to 30 days and evaluated for dimerization by analytical size-exclusion HPLC (SEC) (Figure 3, Table 2). As expected based on prior studies, WT* and P93A* showed no observable dimer formation over the 30 day incubation under these near-physiological conditions. The two variants, P114A* and P114G*, however, showed significant dimer formation within 24 hours. These experiments were repeated for all of the variants with the addition cyclophilin A, a peptidyl prolyl isomerase. In the presence of cyclophilin A no change in dimerization propensity was observed (data not shown). Although the BP-RNase A P114G* dimers formed relatively quickly at 37°C (within 24 hours), dimer formation was also observed at 25°C over the course of several days and at 4°C over the course of several weeks.

Determination of K_d for a dimer-forming variant

In order to determine if the fraction of dimer measured represented an equilibrium population, we monitored two samples of P114G* with the same total protein concentration but different initial dimer populations. Two samples were prepared at 5 mg/mL and incubated at 37°C for several days. One started as a purely monomeric sample, while the other was diluted from a 10 mg/mL sample that contained 20% dimer. As shown in Figure 4, after several days both samples show the same percentage of dimer, suggesting that the system is at equilibrium. It is important to note that under similar conditions WT* does not show any dimer formation and pre-isolated dimers (formed via lyophilization from 50% acetic acid) dissociate very slowly (Figure 4b).

Based on the above results, we estimated a K_d for dimer formation of the P114G* variant by incubating various concentrations of the protein (1.5–22 mg/mL) at 37°C and allowing the system to equilibrate for 3–7 days. The relative concentration of monomer and dimer were analyzed by SEC. Figure 5 shows the resulting plot of $[M]^2$ versus $[D]$, which is well fit by a K_d of 3.66 mM with an $R^2 = 0.999$.

Evaluating for Domain Swapping

While the size exclusion results clearly show dimer formation in the P114G and P114A variants, they do not report on whether or not these dimers are in fact domain-swapped. In order to investigate domain swapping directly, we performed an activity-rescue experiment based on early work by Stein and Moore²². RNase A contains two active site histidines, H12 and H119. Domain swapping between one variant with only H119 and one with only H12 will create a dimer with one active subunit containing both histidines and one with neither, resulting in one active and one dead subunit per dimer (Figure 6). Thus, if domain swapping is the mechanism of dimer formation, mixing these two inactive monomers should result in a reconstituted, active dimer.

We therefore created a set of RNase A variants analogous to our original set, but with an H12A mutation instead of H119A. Mixtures of H119A and H12A variants for each construct (WT, P114G, P114A, and P93A) were incubated at 10 mg/mL total protein at 37°C in 100 mM Tris, pH 8.0 for 24 hours and evaluated for dimerization (as above) and activity (see Materials and Methods). Mixtures of H12A and H119A variants in the background of either P114G or P114A formed dimers and showed RNase A activity, while in the WT and P93A backgrounds no dimers were observed and no activity was detected (Figure 7, Table 2). In other words, only reactions containing a mixture of the active site variants and a mutation at P114 rescued RNase A activity.

C- or N-terminal Domain swapping?

The above experiments clearly demonstrate the formation of domain-swapped dimers under near physiological conditions when P114 is mutated to Ala or Gly. In order to differentiate N-terminal domain-swapped dimers from C-terminal domain-swapped dimers, a similar activity rescue experiment was carried out by mixing the RNase A H119A constructs with a fifteen-residue synthetic peptide encoding the C-terminal exchanged arm and hinge region of RNase A (residues 112–124 and an N-terminal tryptophan). Since the proteins already contain a histidine at position 12 and the peptide contains H119, if the peptide binds in a way that effectively recapitulates the RNase A active site, activity should be rescued. As was observed above in the activity rescue experiments involving two full-length proteins, only mixtures of the C-terminal peptide and protein containing a mutation at P114 rescued activity (Figure 7c, Table 2).

Generalizing the role of cis-P114 as a gatekeeper in homologous RNases

Human pancreatic ribonuclease (RNase 1) is 70.2% identical to BP-RNase A and forms a similar three-dimensional structure²³. At the C-terminus, the two proteins are identical except for a site-specific variation at position 113 and the inclusion of 4 extra residues at the C-terminus of RNase 1. These additional residues do not show significant contacts with the rest of the structure²³, and for the purposes of directly comparing BP-RNase and RNase 1 in this study, these 4 residues were deleted to create a variant herein referred to as R1sWT. Variants were also developed containing mutations of the two homologous *cis*prolines at positions 93 and 114. These variants are referred to as R1sP93A and R1sP114G, respectively.

The swapping propensities of the RNase 1 variant proteins were evaluated using SEC as described above over a time scale from days to several weeks. These studies had to be carried out at 4°C to avoid protein precipitation. Under these conditions, R1sWT and R1sP93A showed no observable dimer formation after several weeks. However, R1sP114G readily forms dimers at 4°C within 24 hours (Figure 8), and appears to dimerize much more readily than BP-RNase A P114G*.

Discussion

Bovine pancreatic RNase A has served as a model system for many structural studies on domain-swapped dimers and a comparison of the crystal structures of monomeric and domain-swapped dimers of BP-RNase A reveal very few obvious differences with the exception of a *cis/trans* isomerization state of the X-Pro bond at P114 at the base of the swapped-hinge region⁴. This observation, coupled with the increased frequency of prolines in hinge regions involved in domain swapping¹³, led us to question if these prolines can act as conformational gatekeepers in the domain swapping mechanism of RNase A. Indeed, mutation of these prolines results in the formation of domain-swapped dimers under physiological conditions. We find that RNases with a mutation at position 114 form a significant fraction of dimer after simple incubation at pH 8.0, whereas variants that retain a proline at position 114 (WT* and P93A*) do not. Moreover, activity rescue assays indicate that the dimers are indeed domain swapped. Our results suggest that the presence or absence of a *cis*-proline in the C-terminal hinge region has a significant effect on RNase A's ability to domain swap.

Cis-Pro114 acts as a conformational gatekeeper in both BP-RNase and RNase 1

The *cis*-proline at position 114 is a conserved feature in several RNases (including BP RNase A, bovine seminal RNase, human pancreatic RNase 1, and RNase A from *Rattus Norvegicus*). Except for BP-RNase A, which shows C-terminal domain swapping after

lyyophilization and other harsh treatments, none of these are known to domain swap at the C-terminus (BS-RNase readily domain swaps at the N-terminus²⁴, and RNase 1 has also been engineered to swap at its N-terminus²⁵). Analogous to our results for BP RNase A, we find that a P114G variant of RNase 1 shows significant dimer formation, whereas WT and P93A RNase 1 remained monomeric, suggesting that our observation of *cis*-P114 as a conformational gatekeeper is likely generalizable to at least these other homologous proteins.

Swapping propensity is not related to global stability

The variants of BP-RNase A that show notable C-terminal domain swapping are also destabilizing. The global stability (ΔG_{unf}) of both P114G* and P114A* is decreased by about 2 kcal/mol (relative to WT*), suggesting that their increased swapping propensity may be the result of global destabilization of the monomers. The variant P93A*, however, also destabilizes the protein by approximately 2 kcal/mol without any notable domain swapping. We therefore do not believe that the noted increased swapping propensity is a direct result of an increase in the population of the unfolded state of these variants.

A likely alternative explanation is that these variants increase the protein's ability to populate an exchange-competent monomeric form and ultimately dimerize via domain swapping. This model is supported by the fact that in the crystal structure of P114G19, the peptide bond between 113 and 114 is *trans* with significant rearrangements in the hydrogen bonding network between the C-terminal β -strand and the core of the protein. This exchange competent form may simply be the *trans* version of the native monomer, or more likely, a partially unfolded intermediate lacking a docked C-terminal strand. Although we have no direct evidence for such a local unfolding event, our peptide rescue experiments support this hypothesis. When the WT*, P114G*, P114A*, and P93A* variants are incubated with a C-terminal peptide composed of residues 112–124 (hinge region and C-terminal β -strand) under conditions similar to those used to initiate domain swapping, only the P114 variants have rescued activity. In order for the C-terminal peptide to restore activity, the interface between the core of the protein and the exchanged C-terminal β -strand must be transiently exposed to allow peptide binding. Since this rescue is specific for the proteins with mutations at position 114, we infer that only these variants undergo the local structural change required to expose the peptide-binding interface.

Studies by Nakano et al. also suggest that the C-terminal peptide can inhibit domain-swapped dimerization²⁶. In these studies, protein dimerization did not occur when WT RNase A was incubated with the C-terminal peptide and lyophilized in acetic acid, suggesting that the peptide binds and blocks docking of an exchanged arm from another monomer in the same structural location, supporting the hypothesis that the C-terminal peptide is capable of binding to RNase A and displacing the covalently attached C-terminal β -strand. However, other aspects of the study by Nakano et al.²⁶ are at odds with our results. The authors report a binding constant between the peptide and WT RNase A (pH 6.7) of 84 ± 38 mM, implying that this peptide can bind effectively to WT RNase A in solution at neutral pH. Our results, however, show that under neutral conditions incubation of the peptide with WT* does not restore activity, suggesting that the peptide cannot displace the C-terminal strand without aid of a mutation at P114. To resolve this discrepancy, we constructed binding curves for the C-terminal peptide and each of our four constructs using the same methodology employed by Nakano et al.²⁶. All four constructs showed a $K_d \sim 200$ mM (data not shown), a value not far from that reported by Nakano et al.²⁶. However, when aliquots of the samples used to construct the binding curves were tested for activity, only the combinations of peptide with the P114 mutants were active, leading us to conclude that the observed changes in intrinsic peptide fluorescence were not reporting on

the peptide displacing the C-terminal β -strand as originally hypothesized, but rather reflect some unknown binding event.

Is the observed domain swapping a kinetic or thermodynamic effect?

Although it is clear that the presence or absence of a *cis*-proline in the hinge region of RNase A affects the formation of the C-terminal domain-swapped dimer, it is unclear if this is because the wild-type dimers are kinetically inaccessible and the variants lower the kinetic barrier, or if the wild-type dimers are thermodynamically unfavorable and the variants simply decrease the K_d to an observable range.

We find that RNase A variants with a *cis*-proline at position 114 do not form measurable dimer even when incubated for up to a month at 37°C. In addition, the purified WT* dimers formed from lyophilization in acetic acid dissociate very slowly at 37°C, retaining a significant population of dimer after a month at 37°C. Together, these results indicate that there is likely a large kinetic barrier between the monomeric and dimeric forms WT* RNase A. This result is in stark contrast to our results with the P114 variants, which readily form dimer under the same conditions, and equilibrate to the same dimer concentration from either the association or dissociation direction. Conversion between monomer and dimer for the P114 mutants is likely at equilibrium, and mutation of P114 to an amino acid that strongly favors a trans conformation significantly lowers the barrier between these two populations. This certainly makes sense, as proline isomerization is often a rate-limiting step in protein folding and unfolding, and substitution of P114 with glycine or alanine may eliminate the need for proline isomerization between the monomer and dimer forms.

In order to obtain direct support of this kinetic model, we attempted to use cyclophilin A, a peptidyl-prolyl isomerase, to catalyze the isomerization of P114 in WT and P93A variants, without any observed effect. While this result does not support a kinetic barrier involving proline isomerization, it does not rule out this model either. Cyclophilin A may not have access to the X-Pro114 bond.

Interestingly, proline isomerization is acid catalyzed²⁷. If isomerization at Pro 114 is a key event in the C-terminal domain swapping of RNase A, this would provide new insight as to why the traditional conditions for dimerization A are so effective. The acetic acid may help catalyze proline isomerization, which, together with the high local concentrations obtained during lyophilization, would promote domain swapping. This is, of course, speculation; there may be other events that affect RNase A during lyophilization that lead to domain swapping.

It should be noted that a previous activity-based study²⁸ suggested that WT RNase A could form dimers under physiological conditions at a total protein concentration of just 12 μ M (0.16 mg/mL). In these experiments, the observed dimerization occurred on the time scale of minutes. It is likely that these dimers are not the same as those studied here, since we observe very slow dimer association and dissociation kinetics, and based on their results we would expect to see significant dimer formation in the wild-type protein at 10 mg/mL.

Although a decrease in a kinetic barrier seems a likely mechanism for the observed dimerization in the proline variants, our results do not exclude the possibility that the observed dimerization may be a thermodynamic effect. While, as discussed above, we can rule out a change in global stability of the monomer as the basis for the change in dimerization, we cannot rule out an effect due to an increase in the stability of the dimer for the variants lacking a proline at position 114. We estimate that our detection limit for dimer formation is ~ 2% dimer, which would correspond to a K_d of 70mM. Thus any dimer

formed with a K_d higher than 70 mM (or K_a less than 14 M^{-1}) would be too unstable for detection.

One potential thermodynamic model for the formation of domain swapped dimer is diagrammed in the scheme below. In this model, the folded monomer exists in equilibrium between *cis* and *trans* at the X-Pro114 bond with an equilibrium constant K_{ct} and a dimerization constant for the *trans* monomer of K_a , as shown in equation 1.

The observed, apparent association constant, K_{app} , will depend on the populations of both N_c and N_t , and can be rewritten in terms of K_a and K_{ct} :

$$K_{app} = [D] / ([N_c] + [N_t])^2 \quad (2)$$

$$K_{app} = K_a / (1 + 1/K_{ct})^2 \quad (3)$$

If we assume that K_a for both WT and P114 variants are the same, then we can use (3) above to compare the effect of *cis/trans* population changes on the observed dimerization (K_{app}). For the P114 variants, $K_{ct} \gg 1$ and $K_{app} \sim K_a$. For the wild type protein, however, the K_{app} will be dramatically decreased due to the effect of K_{ct} .

Our results on the P114G variant give an estimate of K_a ($K_a \sim 273 \text{ M}^{-1}$, $K_d \sim 3.66 \text{ mM}$). Given the above model and our detection limit estimate of 70 mM, we can estimate a minimum K_{ct} for detection of dimer of 0.29. In other words, in order to be able to detect a K_{app} within our detection limits, ~30% of the monomeric protein would have to populate the *trans* conformation at position 114. This significant population should be noticeable by structural methods such as NMR, and such a population has not been observed for WT RNase A. Based on this model, the thermodynamic effect of simply shifting the population of monomeric RNase A from N_c to N_t may account for the increased population of the dimeric form when P114 is altered to an amino acid that strongly favors the *trans* conformation.

Whether the effect of P114 mutations on RNase A dimerization is kinetic or thermodynamic, our results clearly show that the *cis*-proline in the hinge region of RNase A plays an important role in preventing the formation of C-terminal domain swapped dimer. Our results point to proline isomerization as a conformational gatekeeper in oligomerization via domain swapping. These studies provide important insight into the mechanism of domain swapping and, by inference, other similar oligomerization events such as amyloid formation. These variants of RNase A are the first we are aware of that are capable of forming a significant population of domain-swapped dimer in solution under neutral conditions, and provide us with a unique opportunity to probe the molecular mechanism of domain swapping. The next challenge will be to use more sensitive structural techniques, such as NMR and hydrogen exchange, to better characterize the folding events that lead to domain swapping and further elucidate the mechanism of C-terminal domain swapping in RNase A.

Materials and Methods

Plasmid Construction and Protein Purification

A plasmid containing the gene for BP-RNase A was generously donated by D. Eisenberg (with permission from the Raines lab, U. of Wisconsin). The WT BP-RNase A gene from this plasmid was PCR amplified and inserted into a pET22b(+) vector. Quikchange mutagenesis (Stratagene) was used to create eight variants of BP-RNase A: H119A (WT*), P114G/H119A, P114G/H12A, P114A/H119A, P114A/H12A, P93A/H119A, and P93A/H12A.

A plasmid containing the gene for RNase 1 was generously donated by R. Raines. This plasmid was further modified via several rounds of Quikchange mutagenesis (Stratagene) to include a TEV-cleavable 6-His affinity tag. This strategy was also used to incorporate a stop codon at the end of the sequence to truncate the protein by 4 residues, so that the C-terminal sequence would be the same length as that of BP-RNase (R1sWT). All versions of RNase 1 discussed in this paper lack these 4 C-terminal residues. In addition, Quikchange mutagenesis was used to generate mutants of this construct carrying the P93A mutation (R1sP93A) or the P114G (R1sP114G) mutation.

All protein constructs were expressed in BL21-codon + cells on LB media. Both the BP-RNases A and RNases 1 were isolated as previously described²⁹ from inclusion bodies. All BP-RNase A variants were purified via gel filtration chromatography using an Hi-Load Superdex-75 16/60 column on a GE Healthcare AKTA purification system in a 20 mM Tris pH 8.0, 0.5 M NaCl buffer. All RNase 1 variants were purified using a 5 mL HisTrap-HP column from GE Healthcare on a GE Healthcare AKTA purification system. The sample was initially purified via elution with a buffer containing 20 mM Tris pH 8.0, 0.5 M NaCl and 0.5 M imidazole. Following this initial purification, the 6-His tag was cleaved using TEV protease, and the protein was dialyzed into buffer containing 20 mM Tris pH 8.0, 0.5 M NaCl, 3 mM reduced glutathione, and 0.3 mM oxidized glutathione. The sample was then rerun over the HisTrap-HP column, where the cleaved protein eluted as flow through and the His-tagged TEV protease was retained on the column before being bumped off by a high imidazole buffer, as indicated above.

Protein Stability Studies

The global stability of the WT*, P114G/H119A, P114A/H119A, and P93A/H119A constructs were determined using guanidinium chloride denaturation monitored by CD spectroscopy. All samples were prepared in 100 mM Tris pH 8.0 and various concentrations of denaturant and allowed to equilibrate overnight at room temperature. CD signal for each sample was measured at 222 nm, 25°C using an Aviv 410 spectrometer and a 1 cm pathlength cuvette. The data were fit to determine the global stability of unfolding, ΔG_{unf} , and its denaturant dependence, m , under the assumptions that the system is two state and that ΔG_{unf} has a linear dependence on denaturant concentration²¹.

Evaluation of dimerization by HPLC Size Exclusion Chromatography

To initialize dimerization, purified protein was concentrated to 10 mg/mL in 100 mM Tris pH 8.0 and incubated at either 4°C, 25°C, or 37°C for periods of time ranging from 24 hours to 30 days. To detect the presence of dimers, 2.5–10 μ L aliquots were removed and run over a Tosoh Biosciences TSK-G2000SWXL HPLC size exclusion column at 25°C. The mobile phase of the column consisted of 0.2 M sodium phosphate buffer pH 6.7 run at 1.0 mL/min. The column was calibrated using a gel filtration molecular weight standard from Biorad and purified RNase A from Sigma. Percentage dimer was calculated based on peak areas detected via UV absorbance at 220 nm.

To determine whether or not the WT* dimer could equilibrate back to its monomeric form, dimerization was induced by lyophilizing a 20 mg/mL sample in 50% acetic acid. The dimer population was then purified using a Hi-Load Superdex-75 16/60 column on a GE Healthcare AKTA purification system as described above. The dimeric fraction was then concentrated to 0.75 mg/mL and incubated at 37°C. Aliquots were periodically removed and analyzed for presence of dimer using analytical HPLC SEC as described above.

To determine whether RNase A P114G* can readily equilibrate between its monomeric and dimeric forms, two samples were prepared at 5 mg/mL in 100 mM Tris pH 8.0. One sample was prepared from fully monomeric protein and allowed to dimerize at 37°C. The other sample was prepared by diluting a 10 mg/mL sample that had previously been allowed to equilibrate at 4°C for several months. This sample already contained a significant population of dimer, greater than that expected for a sample at the lower concentration of 5 mg/mL. This sample was also incubated at 37°C to allow for dimer dissociation. Both samples were periodically measured for presence of dimer using analytical HPLC SEC as described above over a period of 1–30 days.

In order to determine a K_d of dimerization for BP-RNase A P114G*, a monomeric sample at 22 mg/mL in 100 mM Tris pH 8.0 was serially diluted 1:2 to yield 5 total samples ranging in concentration from 1.5–22 mg/mL. These samples were allowed to equilibrate at 37°C for 3–7 days, and aliquots were periodically measured using analytical HPLC SEC as described above to determine the percentage of dimer present. Samples were determined to be at equilibrium when at least two consecutive measurements consistent dimer percentages ($\pm 1\%$ for either sample). The equilibrium dimer percentage was determined by taking the average of the last three dimer percentage readings (except for the 22 mg/mL sample, which was averaged over the last two readings).

To test for detectable catalysis of proline isomerization, similar experiments were performed combining WT* (H119A) and P93A variants with cyclophilin A. The cyclophilin A was produced recombinantly, purified, and donated by James Fraser. These experiments were performed under the conditions described above, with incubation times ranging from 24 hours to 21 days. Two sets of experiments were performed, one with an RNase A concentration of 730 μ M and cyclophilin A concentration of 10 μ M, and another with an RNase A concentration of 365 μ M and cyclophilin A concentration of 55.6 μ M.

Activity Assays

The presence of rescued activity in the domain-swapped dimers or peptide-bound monomers was detected using activity assays monitoring CMP hydrolysis as described previously³⁰. Assays were performed using 0.1 mg/mL CMP (from Sigma) in 100 mM Tris pH 8.0 and a final RNase A concentration of 100 μ g/mL. The aliquots of BP-RNase A used in the activity assays were taken from the same samples that had been incubated at 37°C as described above and analyzed for presence of dimer via HPLC. Activity was measured using UV spectroscopy monitoring change in absorption at 284 nm. It should be noted that although the total concentration of RNase A in these assays was 100 μ g/mL, the concentration of active protein should only be ~5–10 μ g/mL, since only ~20% of the sample will dimerize and statistically half of those dimers will be inactive homodimers. The samples used to test for rescued activity in RNase A monomers in the presence of the C-terminal peptide contained 31 μ M peptide and 100 μ g/mL RNase A that had been incubated at 25°C for 4 days.

Peptide-Binding Assay

The C-terminal RNase A peptide was synthesized by David King using a peptide synthesizer and has the sequence Ac-WEGNPYVPVHFDASV-OH. This sequence consists of RNase A residues 112–124 with an added N-terminal tryptophan. Fluorescence measurements were taken using a Fluoromax-3 fluorimeter using a 1 cm pathlength cuvette. Tryptophan emission was monitored at 25°C using an excitation wavelength of 280 nm and an emission wavelength of 380 nm. Samples were prepared in 100 mM Tris pH 8.0 with a constant peptide concentration of 313 μ M. To construct binding curves, a sample was prepared with an initial RNase A concentration of ~10 mg/ mL (~750 μ M) and was allowed to equilibrate for 20 minutes. This sample was then serially diluted into solutions containing only buffer and peptide in order to maintain a constant peptide concentration while titrating down the RNase A concentration. Subsequent samples were allowed to equilibrate for 10 min, and equilibration of each sample was monitored via a kinetic trace of Trp emission. An average of the last 300 seconds of each kinetic trace was used to determine the equilibrium fluorescence signal of each serially diluted data point. These data were then used to construct a binding curve fit to a 2-parameter hyperbolic decay curve to determine the apparent K_d of peptide binding.

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Figure 1. Structures of the monomer and C-terminal domain-swapped dimer of Ribonuclease A
a) PDB ID 1RTB. In monomeric RNase A, Pro 114 adopts a cis conformation b) PDB ID 1F0V. In the C-terminal domain-swapped dimer of RNase A, Pro 114 adopts a trans conformation, resulting in the extension of the hinge between the protein core and the exchanged C-terminal arm. C) PDB ID 1KH8. In the P114G variant of RNase A, the backbone adopts a trans conformation.

Figure 2. Comparison of the global stability of the four constructs studied

Denaturation melts were performed using GdmCl in 100 mM Tris pH 8.0 at 25°C. WT* (filled circles) P114A* (open circles), P114G* (closed triangles), and P93A* (open triangles).

Figure 3. HPLC analytical size exclusion chromatography detects presence of dimer in BP-RNase A variants

These chromatograms are representative of the results achieved when the BP-RNase A variants studied were incubated in 100 mM Tris pH 8.0 at approximately 10 mg/mL total protein for up to 30 days at 25°C or 37°C, or up to several months at 4°C. The Pro 114 mutants (P114A and P114G) show a dimer peak that elutes from the column ~1.2 minutes before the monomer, while constructs that contain the WT P114 have only a monomeric peak.

Figure 4. Dimer association and dissociation

a) The dimer populations of two samples of RNase A P114G* at 5 mg/mL were monitored as a function of time at 37°C. The dimer association sample started as pure monomer, while the dissociation sample was diluted 1:2 from a 10 mg/mL sample that had equilibrated to 20% dimer. This sample then relaxes to the same dimer population as the association sample as some of the dimer population dissociates over time. b) The WT* (H119A) dimer was isolated and concentrated to a concentration of 27.6 μ M (0.751 mg/mL total protein). The sample was then incubated at 37°C and dimer dissociation was allowed to proceed. A population of 14.5 % dimer remained even after a month-long incubation period.

Figure 5. Determination of dissociation constant for RNase A P114G dimers

RNase A P114G was incubated at various concentrations in 100 mM Tris pH 8.0 and allowed to equilibrate for several days. The slope of the linear fit is equal to the dissociation constant (K_d) for dimer formation, 3.66 mM. $R^2 = 0.9997$.

Figure 6. Domain swapping between two inactive mutants of RNase A restores the RNase A active site

The two active site residues of RNase A are His 119 (dark blue) and His 12 (red). If one of these residues is mutated, the resulting monomer is inactive. However, upon domain swapping, if either the C terminal (shown) or N-terminal arm is exchanged, the resulting dimer consists of one subunit that contains both active site histidines, and one subunit that contains none. Monomeric structure PDB ID 1RTB, dimeric structure PDB ID 1F0V.

Figure 7. Only constructs that domain swap regain enzymatic activity

RNase activity was measured via hydrolysis of CMP monitored via absorbance at 284 nm. The slope of these progress curves is directly proportional to the rate constant, k of the reaction. a) Mixtures of WT* H119A and H12A mutants (squares) and P93A/H119A and P93A/H12A mutants (x's) are not active, while mixtures of P114A/H119A and P114A/H12A mutants (circles) and P114G/H119A and P114G/H12A mutants (triangles) do show reconstituted activity. b) Individual active site mutants P114A/H119A (empty circles), P114A/H12A (filled circles), P114G/H119A (empty triangles), and P114G/H12A (filled triangles) are not active, and their hydrolysis curves are similar to those of a solution containing CMP substrate only (curve indicated by asterisks). c) All 4 constructs, WT*

(H119A, squares), P93A/H119A (diamonds), P114A/H119A (circles), and P114G/H119A (triangles) were incubated in the presence of a peptide containing the C-terminal residues 112–124, only the two P114 mutants showed rescued activity in the presence of the peptide.

Figure 8. HPLC analytical size exclusion chromatography detects dimer in human pancreatic ribonuclease (RNase 1) variants

These chromatograms are representative of the results achieved when the RNase 1 variants studied were incubated in 100 mM Tris pH 8.0 at approximately 10 mg/mL total protein for up to 30 days at 4°C or several days at 25°C or 37°C. The R1sP114G mutant shows a dimer peak that elutes from the column ~1.1 minutes before the monomer, while constructs that contain P114 have only a monomeric peak.

Table 1

The stabilities (ΔG_{unf}) for variants of BP-RNase A at 25°C.

Construct	ΔG_{unf} (kcal/mol)	m (kcal/mol•M)
WT*	8.9 $\pm$ 0.5	3.0
P114G/H119A	6.7 $\pm$ 0.4	3.2
P114A/H119A	6.9 $\pm$ 0.3	3.1
P93A/H119A	6.9 $\pm$ 0.4	3.1

Table 2

Summary of activity assays for BP-RNase A variant mixtures, shaded boxes indicate variants that dimerize.

	H119A	H12A	H119A and H12A	H119A and C-terminal peptide
WT	–	–	–	–
P93A	–	–	–	–
P114A	–	–	+	+
P114G	–	–	+	+