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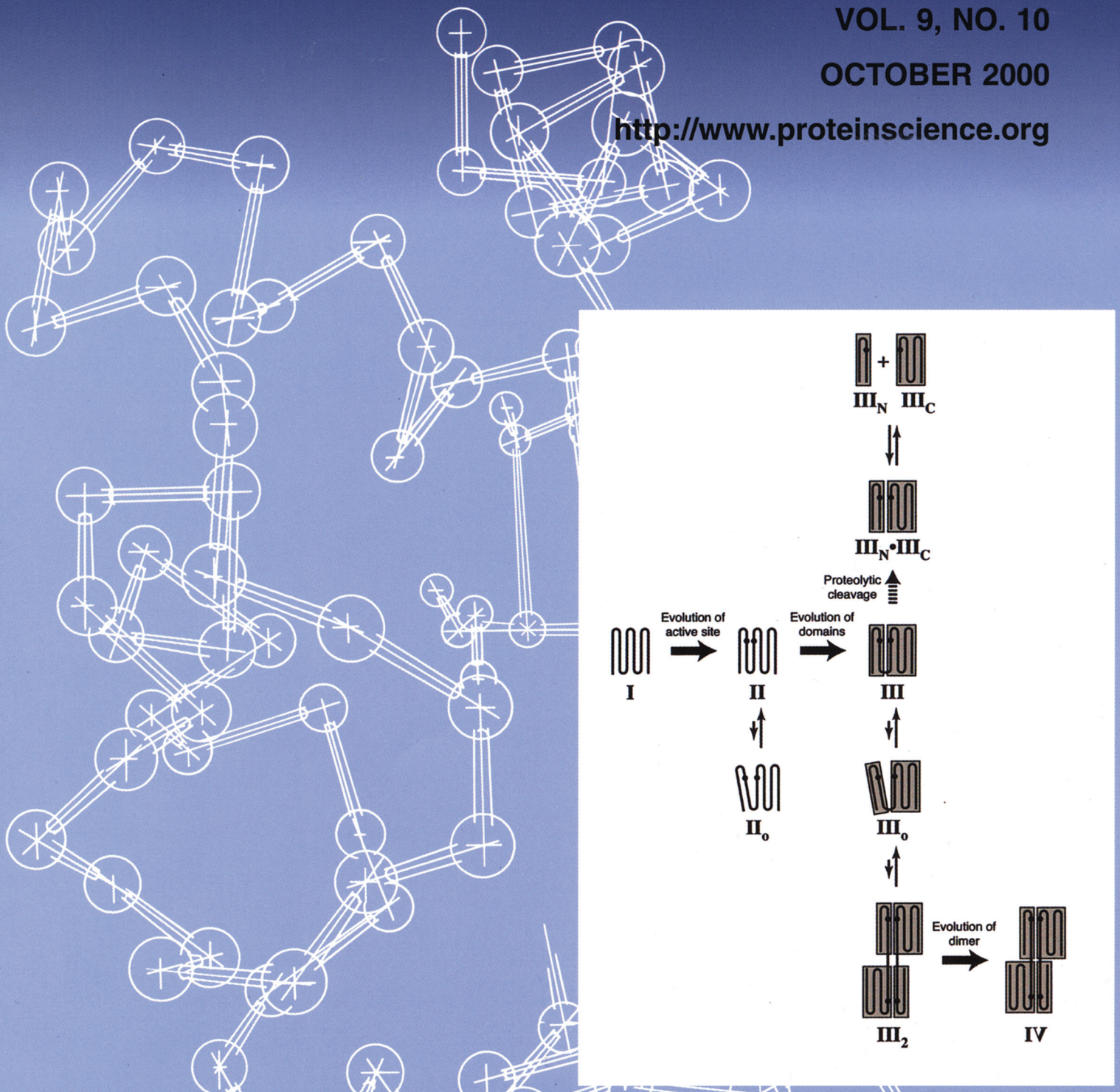

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Dimer formation by a "monomeric" protein

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Abstract

Dimeric proteins can arise by the swapping of structural domains between monomers. The prevalence of this occurrence is unknown. Ribonuclease A (RNase A) is assumed to be a monomer near physiological conditions. Here, this hypothesis is tested and found to be imprecise. The two histidine residues (His12 and His119) in the active site of RNase A arise from two domains (S-peptide and S-protein) of the protein. The H12A and H119A variants have 10^5-fold less ribonucleolytic activity than does the wild-type enzyme. Incubating a 1:1 mixture of the H12A and H119A variants at pH 6.5 and 65 °C results in a 10^3-fold increase in ribonucleolytic activity. A large quantity of active dimer can be produced by lyophilizing a 1:1 mixture of the H12A and H119A variants from acetic acid. At pH 6.5 and 65 °C, the ribonucleolytic activity of this dimer converges to that of the dimer formed by simply incubating the monomers, as expected for a monomer–dimer equilibrium. The equilibrium dissociation constant for the dimer is near 2 mM at both 65 and 37 °C. This value of K_d is only 20-fold greater than the concentration of RNase A in the cow pancreas, suggesting that RNase A dimers exist in vivo. The intrinsic ability of RNase A to form dimers under physiological conditions is consistent with a detailed model for the evolution of homodimeric proteins. Dimers of "monomeric" proteins could be more prevalent than is usually appreciated.

Keywords: composite active site; dimer; domain swapping; molecular evolution; ribonuclease A

The assembly of oligomeric proteins from monomers by domain swapping is a recurrent theme (Bennett et al., 1995; Schlunegger et al., 1997). Bovine seminal ribonuclease (BS-RNase) is a quintessential example of a domain-swapped dimer (D'Alessio et al., 1997; D'Alessio, 1999a, 1999b). Although ribonuclease A (RNase A) and BS-RNase share a similar three-dimensional structure as well as more than 80% of their amino acid residues, only BS-RNase is known to form a domain-swapped dimer naturally (Fig. 1A) (Piccoli et al., 1992; Mazzarella et al., 1993). BS-RNase can also form a domain-swapped tetramer (Adinolfi et al., 1996).

RNase A can form domain-swapped dimers by an artificial procedure—lyophilization from a concentrated solution in 50% acetic acid (Crestfield et al., 1962, 1963). This dimer has been shown to have two different conformers (Gotte & Libonati, 1998; Sorrentino et al., 2000). The structure of one of these conformers has been determined (Liu et al., 1998) and is distinct from that of the BS-RNase dimer (Fig. 1B). Dimeric RNase A is thermally unstable, dissociating to monomers irreversibly upon incubation at high temperature (Crestfield et al., 1962).

His12 and His119 are active-site residues near the N- and C-termini, respectively, of RNase A. The H12A and H119A variants have 10^5-fold lower ribonucleolytic activity than does the wild-type enzyme (Thompson & Raines, 1995). Dimerization of these variants by domain swapping should result in composite active sites and the regain of ribonucleolytic activity (Fig. 2). Hence, an assay of enzymatic activity could be used to assess readily and quantitatively the extent of domain swapping. Here, we use this method to analyze the thermodynamics and kinetics of dimer formation by RNase A. Most significantly, we find that RNase A spontaneously forms domain-swapped dimers *near neutral pH*. This discovery of a pre-evolved ability for domain swapping in a monomeric protein enables us to propose a detailed model for the evolution of dimeric proteins.

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Results

Detection of dimers

Protein complementation by enzyme variants has been recognized as a useful tool to study the structure of multimeric enzymes with composite active sites (Larimer et al., 1987; Wente & Schachman, 1987; Tobias & Kahana, 1993; Li et al., 1997) or multiple domains (van Gent et al., 1993; Kao et al., 1996; Witkowski et al., 1996). Chemical modification of RNase A and BS-RNase had been used previously to show the existence of composite active sites by the formation of active dimers from inactive monomers (Crestfield et al., 1963; Piccoli et al., 1992). Because of the residual catalytic activity remaining after incomplete modification and purification,

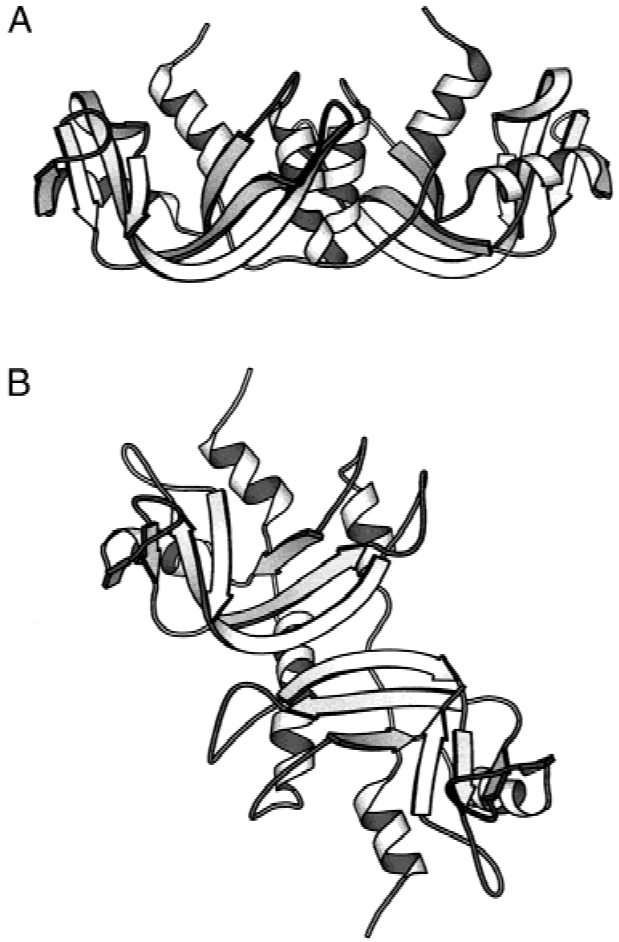

Fig. 1. Ribbon diagram of bovine seminal ribonuclease [(**A**) Protein Data Bank entry 1BSR (Mazzarella et al., 1993)] and the ribonuclease A dimer formed by lyophilization [(**B**) Protein Data Bank entry 1A2W (Liu et al., 1998)]. The diagram was made with the program MOLSCRIPT (Kraulis, 1991).

chemically modified enzymes do not allow for quantitation of domain swapping. Using H12A RNase A and H119A RNase A, along with sensitive assays for ribonucleolytic activity, allowed us to monitor accurately the dimerization of RNase A.

Enzymatic activity

Steady-state kinetic parameters for the cleavage of poly(cytidylic acid) [poly(C)] by the H12A RNase A·H119A RNase A dimer formed by lyophilization from 50% acetic acid are listed in Table 1. Assays were performed at pH 7.0 instead at pH 6.0 (which is more typical), because the dimer was observed to lose its ribonucleolytic activity slowly at pH 6.0. For comparison, steady-state kinetic parameters for monomers of wild-type RNase A was also determined under the same conditions (Table 1). The values of k_{cat} and k_{cat}/K_M for the H12A RNase A·H119A RNase A dimer are (39 ± 4)% and (31 ± 2)% of those for the wild-type monomer, respectively. These values are close to 25%, the relative activity expected from a theoretical 50% yield of dimers with 50% of the activity of wild-type RNase A.

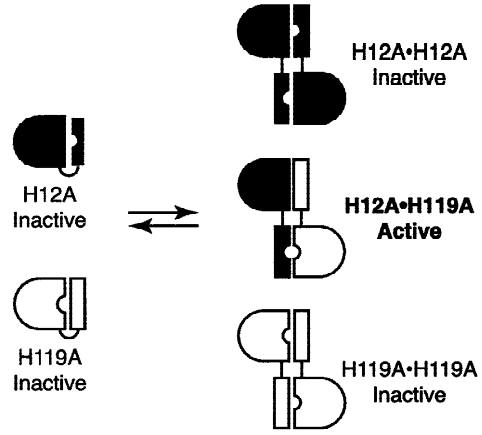

Fig. 2. Scheme for the dimerization of H12A ribonuclease A and H119A ribonuclease A. RNase A is depicted as a complex of the S-peptide and S-protein domains connected by a flexible loop. The location of the histidine residues that are absent in the H12A (black) and H119A (white) variants are denoted with an indent. In the H12A RNase A·H119A RNase A dimer, only one active site is intact.

Dimer dissociation

The dimer of H12A RNase A and H119A RNase A formed by lyophilization lost its catalytic activity rapidly when incubated at 65 °C (Fig. 3A). In only 2 min, the specific activity decreased from 58 s^{-1} to 0.29 s^{-1}, which is a 99.5% decrease. Surprisingly, the remaining activity increased slowly and converged to 0.82 s^{-1}, which is 10^3-fold higher than that of monomers of H12A RNase A or H119A RNase A. This convergence indicates that the dimer does not convert to monomer completely upon incubation at 65 °C.

The conformational stability of the dimer was also determined at 37 °C. The dimer was stable at 37 °C (Fig. 3B). After a slight initial decrease, the activity remained constant for 90 min.

Monomer–dimer equilibrium at neutral pH

To probe for the existence of a monomer–dimer equilibrium near neutral pH, an equimolar mixture of H12A RNase A and H119A RNase A was prepared and incubated at pH 6.5 and 65 °C. The catalytic activity of the mixture increased rapidly (Fig. 3A). The specific activity reached 0.71 s^{-1} in 2 min and did not change until 90 min. The average of the last three specific activities was

Table 1. *Steady-state kinetic parameters for the cleavage of poly(cytidylic acid) by wild-type ribonuclease A and the dimer of the H12A and H119A variants formed by lyophilization from 50% (v/v) acetic acid* [a]

Ribonuclease A	k_{cat} (s^{-1})	k_{cat}/K_M (10^6 $M^{-1}s^{-1}$)	K_M (mM)
Wild-type	880 ± 80	1.6 ± 0.1	0.55 ± 0.06
Dimer	340 ± 20	0.50 ± 0.02	0.68 ± 0.05

[a] Parameters were calculated from nonlinear regression analysis of data collected at pH 7.0 and 25 °C.

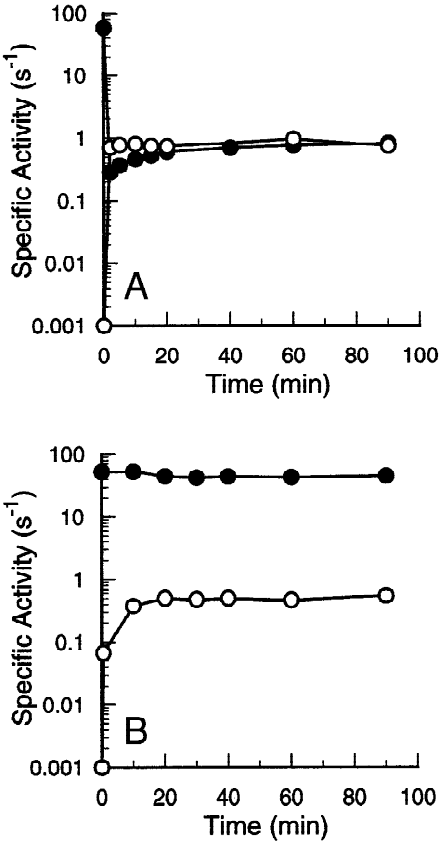

Fig. 3. Dissociation and formation of a dimer of H12A ribonuclease A and H119A ribonuclease A at (**A**) 65 °C and (**B**) 37 °C. Dimers were formed by lyophilization from 50% acetic acid (●), or by mixing at pH 6.5 (○). Specific activities were determined by monitoring catalysis of poly(C) cleavage. The specific activity of the mixture of H12A RNase A and H119A RNase A at 0 min is the average of the specific activity of each variant.

$(0.76 \pm 0.06)\ s^{-1}$. This value is within error of $0.82\ s^{-1}$, the activity remaining after the thermal inactivation of the dimer formed by lyophilization. This convergence to the same activity by heating the dimer to 65 °C or mixing the monomers at 65 °C indicates that a monomer–dimer equilibrium does indeed exist. By using $SA_{obs} = (0.76 \pm 0.06)\ s^{-1}$ as the observed specific activity at equilibrium, $SA_{ref} = (58 \pm 3)\ s^{-1}$ as the maximal specific activity (vide supra), and $[M]_{total} = 12\ \mu M$ as the total concentration of H12A RNase A and H119A RNase A, the concentration of dimer at equilibrium was estimated to be $[D] = (0.079 \pm 0.007)\ \mu M$ by Equation 1.

$$[D] = \frac{1}{2}\left(\frac{SA_{obs}}{SA_{obs}}[M]_{total}\right). \quad (1)$$

With this concentration, the equilibrium dissociation constant was determined to be $K_d = (1.8 \pm 0.1)$ mM by Equation 2 (Table 2).

$$K_d = \frac{([M]_{total} - 2[D])^2}{[D]}. \quad (2)$$

To determine the equilibrium dissociation constant at physiological temperature, a mixture of H12A RNase A and H119A RNase A was likewise incubated at 37 °C (Fig. 3B). The activity of this mixture also increased sharply and reached a specific activity of $(0.50 \pm 0.03)\ s^{-1}$, which is similar to that of the mixture incubated at 65 °C. The concentration of dimer at equilibrium was calculated to be $[D] = (0.052 \pm 0.004)\ \mu M$ by Equation 1. The equilibrium dissociation constant was determined to be $K_d = (2.7 \pm 0.2)$ mM by Equation 2 (Table 2). The activity of the mixture did not change significantly after 20 h of incubation (data not shown).

Table 2. *Properties of the dimer formed by H12A ribonuclease A and H119A ribonuclease A pH 6.5*

Method	Specific activity (s^{-1})[a]	[Dimer] (μM)[b]	K_d (mM)[b]
Quenched on ice after incubation at 65 °C	0.76 ± 0.06	0.079 ± 0.007	1.8 ± 0.1
Incubated at 37 °C	0.50 ± 0.03	0.052 ± 0.004	2.7 ± 0.2

[a] Specific activities are expressed as the mean (±SE) of last three time points in Figures 3A and 3B.
[b] The values of [Dimer] and K_d were calculated by using Equations 1 and 2.

Discussion

Protein complementation by H12A and H119A variants

The dimer formed by lyophilization loses its catalytic activity rapidly at 65 °C (Fig. 3A), as expected from the reported instability of the dimer (Crestfield et al., 1962). Rapid inactivation can be a useful property for practical applications of ribonucleases. The high thermal stability and folding reversibility of RNase A make it impractical to inactivate the enzyme by heat treatment. Thus, modulation of RNase A activity in vitro has been a problem attacked by both inhibitor design (Hummel et al., 1987; Stowell et al., 1995; Russo & Shapiro, 1999) and protein engineering (Messmore et al., 2000). The dimer of H12A RNase A and H119A RNase A allows for facile regulation of ribonucleolytic activity in vitro. Rapid inactivation can be achieved simply by heating to 65 °C without the addition of any chemical reagent.

A dimer of RNase A at neutral pH

The formation of a dimer in a mixture of the H12A and H119A variants demonstrates the existence of a monomer–dimer equilibrium, even at neutral pH. This dimer has $K_d = (1.8 \pm 0.1)$ mM at 65 °C and $K_d = (2.7 \pm 0.2)$ mM at 37 °C (Table 2). A dimer of wild-type RNase A could have a slightly different K_d from those measured with the H12A RNaseA·H119A RNase A dimer. The H12A RNaseA·H119A RNase A dimer has one intact active site (with His12 and His119; Fig. 2) and one impaired active site (with Ala12 and Ala119). The T_m (which is the temperature at the midpoint of the thermal transition between native and denatured states) of the H12A/H119A variant of RNase A is lower by 9 °C than that of the wild-type enzyme (T.A. Klink & R.T. Raines, unpubl. obs.). In other words, the presence of Ala12 and Ala119 diminishes conformational stability. Thus, it is reasonable to expect that the K_d for a dimer of wild-type RNase A may actually be *less* than that for

the H12A RNase A·H119A RNase A dimer. Nevertheless, dimerization of wild-type RNase A near neutral pH had never been observed previously, presumably because its K_d value is too high to allow for its detection by conventional analytical methods (e.g., gel filtration chromatography, analytical ultracentrifugation, calorimetry, and so forth).

The dimer formed near neutral pH seems to be distinct from that formed by lyophilization from 50% acetic acid. The dissociation of the neutral-pH dimer at 37 °C is markedly fast compared with that of the dimer formed by lyophilization from acetic acid (Fig. 3B). The monomer–dimer equilibrium can be written as $A + B \rightleftarrows AB$, with an association rate constant of k_a and a dissociation rate constant of k_d. The rate of dimer formation can be written as

$$\frac{d[AB]}{dt} = k_a[A][B] - k_d[AB]. \quad (3)$$

Because the initial concentration of homodimers in each solution of monomer is negligible, [A] and [B] are approximately equal to $[A]_{total}$ and $[B]_{total}$, respectively. By solving differential Equation 3, [AB] can be written as

$$[AB] \approx \frac{[A]_{total}[B]_{total}}{K_d}(1 - e^{-k_d t}). \quad (4)$$

When the dimer formed by lyophilization dissociates, the initial contribution of $k_a[A][B]$ in Equation 3 is negligible. At short times, [AB] can be written as

$$[AB] \approx [AB]_0 e^{-k_d t}. \quad (5)$$

If the dimer formed at neutral pH has the same structure as the dimer formed by lyophilization, then the two dimers should have the same value of k_d. Nonlinear regression analysis of the data in Figure 3B with Equation 4 (association) and Equation 5 (dissociation), results in disparate values of k_d. The dimer formed at neutral pH has $k_d = (0.15 \pm 0.03)\ s^{-1}$, whereas the dimer formed by lyophilization has $k_d = (0.002 \pm 0.001)\ s^{-1}$. Thus, the dimer formed at neutral pH is distinct from the dimer formed by lyophilization. This result is consistent with the observation of an initial dip in ribonucleolytic activity when the dimer formed by lyophilization from 50% acetic acid was incubated at 65 °C (Fig. 3A). The residual activity initially declines (to $0.29\ s^{-1}$) but slowly recovers (to $0.82\ s^{-1}$). Apparently, the dimer formed by lyophilization (D) dissociates to monomers (M) first, then forms a dimer with a different structure (D*) as in Equation 6.

$$D \rightarrow 2M \rightleftarrows D^*. \quad (6)$$

The dimer formed by lyophilization has been reported to have two distinct conformers (Gotte & Libonati, 1998; Sorrentino et al., 2000). The above kinetic analysis shows that the dimer formed at neutral pH is distinct from that of both conformers formed by lyophilization (Table 3).

The dimer formed by RNase A at neutral pH could have a conformation similar to that of the BS-RNase dimer (Fig. 1A). The monomers of BS-RNase are cross-linked by two intermolecular disulfide bonds (D'Alessio, 1995, 1999a, 1999b; Di Donato et al., 1995). With these disulfide bonds intact, the domain-swapped dimer (M × M) has a free energy equivalent to that of the covalently linked dimer without domain swapping (M = M) (Piccoli et al., 1992; Kim et al., 1995). The formation of a BS-RNase-like dimer by RNase A has a precedent. The K31C/S32C variant of RNase A forms two intermolecular disulfide bonds and swaps its S-peptide domain in the resulting dimer (Di Donato et al., 1995; Ciglic et al., 1998).

Table 3. *Stability and structure of dimers of ribonuclease A*

Dimers	Conformational stability	Structure
Dimer formed by lyophilization (Conformer I[a])	Metastable	Unknown
Dimer formed by lyophilization (Conformer II[a])	Metastable	Liu et al. (1998)
Dimer formed at neutral pH	Unstable	Unknown

[a]The designation is from Sorrentino et al. (2000).

Domain swapping has been shown to endow BS-RNase with distinct biochemical and physiological characteristics, such as allostery (Piccoli et al., 1992) and cytotoxicity (Cafaro et al., 1995; Kim et al., 1995). Due to the structural similarity between BS-RNase and RNase A, engineering RNase A to form dimers like BS-RNase has also been used to endow new biological functions (Di Donato et al., 1994; Cafaro et al., 1998) or to explore the molecular evolution of BS-RNase (Di Donato et al., 1995; Ciglic et al., 1998). RNase A as well as its human homolog have been engineered to form dimers, and the cytotoxicity of those variants approaches that of BS-RNase (Cafaro et al., 1998; Piccoli et al., 1999). Given that the engineered dimeric structure confers cytotoxicity to RNase A, the endogenous concentration of dimeric RNase A in vivo is of significant interest. The concentration of RNase A in bovine pancreas has been estimated to be 0.1 mM (Barnard, 1969). With the assumption that the K_d of 2.7 mM determined here for dimer dissociation at 37 °C is still valid in vivo, the concentration of domain-swapped RNase A dimer in bovine pancreas is 3 μM. This value is comparable to the IC_{50} value of BS-RNase for transformed cell lines (Kim et al., 1995). This finding could have important implications in understanding the biological role of RNase A.

Evolution of homodimeric proteins

The evolution of homodimeric proteins is a topic of much current interest in molecular evolution (Bennett et al., 1995; Ciglic et al., 1998). Because multiple mutations are necessary to provide a complimentary intermolecular interface, the mechanism for the evolution of a stable dimeric protein is enigmatic. Domain swapping has been proposed to explain how an intermolecular interface can evolve without changing multiple residues (Bennett et al., 1995). In the domain swapping model, a monomeric protein swaps one of its domains with another monomer to form a homodimer. Then, residues can be replaced to stabilize the nascent dimer.

The intermolecular interface in a domain-swapped homodimer has two components. A "C-interface" is the intermolecular interface that evolves from the *closed* intramolecular interface in each monomer. An "O-interface" is the new intermolecular interface, which was an *open* surface in each monomer. Hence, the evolution

of a domain-swapped homodimer uses a pre-evolved interface in the monomer. Domain swapping of monomeric proteins under harsh, nonphysiological conditions has been proposed as evidence for the use of this pre-evolved interface. For example, RNase A dimerizes upon lyophilization from 50% acetic acid (Crestfield et al., 1962), and diphtheria toxin dimerizes upon freezing in phosphate buffer (Carroll et al., 1988). Our detection of an RNase A dimer at neutral pH demonstrates that RNase A has an intrinsic ability to form a domain-swapped dimer, even without harsh treatment.

The dimerization of RNase A also has an interesting implication for the evolution of composite active sites. As mentioned above, protein complementation experiments have been used to identify proteins with composite active sites (Larimer et al., 1987; Wente & Schachman, 1987; Tobias & Kahana, 1993; Li et al., 1997). The fusion of single-domain proteins has been proposed as a mechanism for the evolution of composite active sites (Bennett et al., 1995). According to this proposal, an active site develops at a domain interface after a primitive monomer with two domains is formed by the fusion of genes encoding two single-domain proteins.

We propose an alternative mechanism for the evolution of homodimers with composite active sites. Our mechanism is shown in Figure 4. This mechanism is based on our detection of an RNase A dimer at neutral pH, the known ability of the S-protein and S-peptide fragments to form a stable noncovalent complex (Richards, 1955; Richards & Vithayathil, 1959), and physicochemical considerations.

We begin with protein **I** (Fig. 4), which is a primitive protein that has no biological activity. An active site evolves by natural selection, to give protein **II**. The evolution of the active site is likely to destabilize the protein for the following reasons. First, active-site residues tend to be hydrophilic so as to provide the Brønsted acids, Brønsted bases, nucleophiles, hydrogen bonds, and Coulombic interactions needed for function (Warshel, 1998). The evolution of a hydrophilic active site creates a solvent-accessible concave surface, which is likely to be less favorable to conformational stability than a hydrophobic core. Second, active-site residues can have pK_a values that differ by several units from their intrinsic pK_a values (for a dramatic example, see Highbarger et al., 1996). Conformational stability must be sacrificed to perturb the pK_a values of these residues. Finally, proteins often have clusters of cationic or anionic residues at their active sites [e.g., the cationic residues of RNase A (Fisher et al., 1998a, 1998b)] to enhance Coulombic interactions with their ligand (e.g., the anionic phosphoryl groups of RNA). This clustering is unfavorable to conformational stability because of repulsive Coulombic forces (Grimsley et al., 1999; Loladze et al., 1999; Perl et al., 2000; Spector et al., 2000). Hence, the evolution of an active site likely destabilizes a protein. Indeed, when His119 of RNase A is mutated to an alanine residue, the conformational stability of the protein is increased by 0.3 kcal/mol at pH 6.0 and 1.1 kcal/mol at pH 1.2 (Quirk et al., 1998). This destabilization by active-site residues allows the protein to open along the interface defining the active site to form protein $\mathbf{II_o}$. The evolution of an active site could thus define the domain structure of a protein.

Protein **II** is vulnerable to denaturation via protein $\mathbf{II_o}$. The open conformation of protein $\mathbf{II_o}$ has fewer favorable intramolecular interactions than does the closed conformation of protein **II**. Protein **III** and protein $\mathbf{III_o}$ have evolved stable domains without compromising the active site, as depicted by the shaded boxes in Figure 4. The stabilized domains could form a noncovalent complex, protein $\mathbf{III_N}$·protein $\mathbf{III_C}$, after proteolytic cleavage between the two domains. The individual domains, protein $\mathbf{III_N}$ and protein $\mathbf{III_C}$, could even be stable (or metastable) independently.

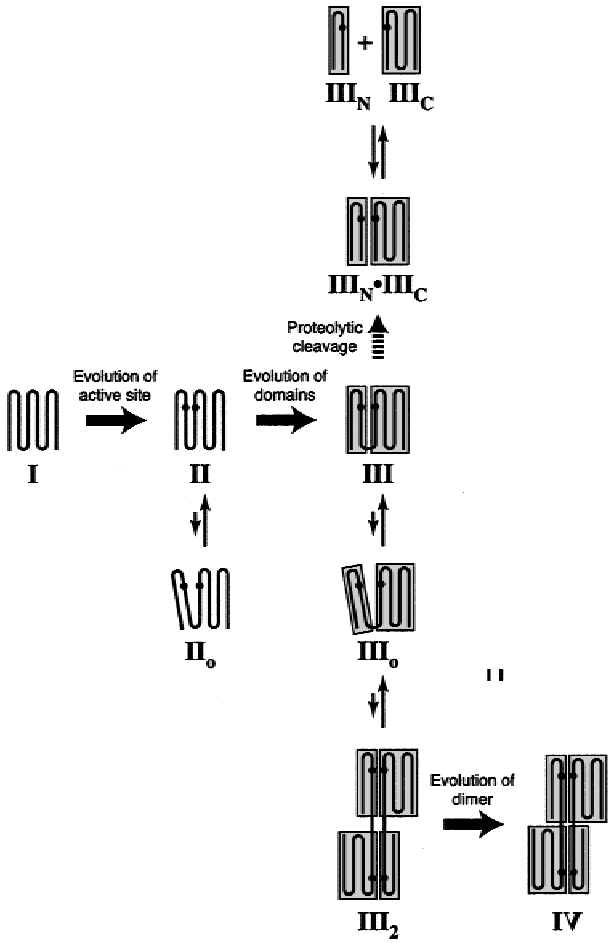

Fig. 4. Putative mechanism for the evolution of a domain-swapped dimer with composite active sites. The primitive monomer does not have an active site (protein **I**). The evolution of the active site (•) destabilizes the protein, allowing equilibration between an intact conformation (protein **II**) and an open conformation (protein $\mathbf{II_o}$). Stable domains evolve to increase conformational stability (protein **III**). In a side reaction, proteolytic cleavage between the domains results in a noncovalent complex (protein $\mathbf{III_N}$·protein $\mathbf{III_C}$) that dissociates into its two components (protein $\mathbf{III_N}$ and protein $\mathbf{III_C}$). The protein with stable domains (protein **III**) forms a primitive domain-swapped dimer (protein $\mathbf{III_2}$). A stable dimer evolves by favorable changes in the dimeric interface (protein **IV**). The steps in this mechanism are shown to be sequential, but could also occur in concert.

RNase S is an example of the protein $\mathbf{III_N}$·protein $\mathbf{III_C}$ complex. S-Protein, which is an example of protein $\mathbf{III_C}$, has a partially disordered but native-like structure (Graziano et al., 1996). Disulfide bonds seem to be responsible for the conformational stability of S-protein, as S-protein cannot form correct disulfide bonds once it is reduced in the absence of S-peptide (Harber & Anfinsen, 1961), which is an example of protein $\mathbf{III_N}$. S-Peptide also tends to fold into its native conformation, an α-helix (Shoemaker et al., 1987).

The equilibrium between protein **III** and protein $\mathbf{III_o}$ allows for the formation of protein $\mathbf{III_2}$, which is a primitive domain-swapped dimer with composite active sites but a high K_d. The unstable

RNase A dimer formed near neutral pH is an example of protein **III$_2$**. Lyophilization of RNase A from 50% acetic acid induces dimerization because the acidic pH increases the population of the open conformation, protein **III$_o$**; and the lyophilization process increases the concentration of protein, which favors its dimerization to form protein **III$_2$**. Dehydration could also enhance dimer formation by desolvating the C- and O-interfaces, and thereby favoring intermolecular association. Lyophilization from 50% acetic acid is likely to produce dimers in several different conformations, but only the metastable conformers are observed routinely (Gotte & Libonati, 1998).

Domain swapping provides a dimerization interface without the need for multiple mutations in a monomer (Bennett et al., 1995). Still, domain swapping alone is unlikely to endow a dimer with stability. Swapping intramolecular interactions for identical intermolecular interactions in a domain-swapped dimer is likely to be isoenthalpic, and dimerization itself has a substantial entropic cost. If there were a phenotypic selection for the dimer, then its K_d could become lower through mutations that stabilize the dimer (e.g., development of an O-interface or evolution of intermolecular disulfide bonds) or destabilize the monomer (e.g., deletion of residues between the two domains). The efficacy of this latter strategy was demonstrated recently with human pancreatic ribonuclease, which forms a homodimer with $K_d = 1.5\ \mu$M upon removal of five residues in the loop that links the S-peptide and S-protein domains (Russo et al., 2000). The result is protein **IV**, which is a stable domain-swapped dimer.

The role of domain swapping in the dimerization of BS-RNase has been controversial (D'Alessio, 1995, 1999a, 1999b). If the integrity of the BS-RNase dimer is paramount, then the intermolecular disulfide bonds are most responsible for dimerization. After all, the dissociation energy of a disulfide bond far exceeds that of any known noncovalent interaction. Although domain swapping plays only a minor role in the stabilization of dimeric BS-RNase, it does dictate the location of the dimerization interface. Moreover, the cytotoxicity of BS-RNase originates from domain swapping, and not from the intermolecular disulfide bonds (Cafaro et al., 1995; Kim et al., 1995).

The lack of evidence for unstable dimers, such as protein **III$_2$** (Fig. 4), had suggested that stable dimers evolve directly from stable monomers. In contrast, we have discovered that RNase A has a predisposition [perhaps even a preadaptation (Gould, 1977)] to form a dimer, albeit an unstable one, at neutral pH. The intrinsic ability of RNase A to form an unstable dimer indicates that evolution of a dimeric quaternary structure can be a continuous process. Assigning a particular mutation as being responsible for dimerization in such a continuous process would be arbitrary. Proteins have been subjected to natural selection as a result of the evolutionary pressures on the organisms that contain them (Burbaum et al., 1989). Hence, it would be most appropriate to ask: Which mutation provided a selectable phenotype for dimerization?

Materials and methods

Protein production and purification

Wild-type RNase A and its H12A and H119A variants (Thompson & Raines, 1995) were produced in *Escherichia coli* and purified as described elsewhere (delCardayré & Raines, 1994; Fisher et al., 1998b) with the following modifications. Inclusion bodies were resuspended in a minimal volume of solubilization buffer, which was 20 mM Tris-HCl buffer (pH 8.0) containing guanidine-HCl (7 M), EDTA (10 mM), and dithiothreitol (DTT) (0.10 M). This suspension was diluted 10-fold by adding 20 mM acetic acid slowly and with stirring. At this step, the dark brown solution turned turbid because of the precipitation of impurities. Reduced RNase A was still soluble after the dilution. The precipitant was removed by centrifugation for 30 min at 15,000 $\times$ *g*. The supernatant from the centrifugation was dialyzed against 20 mM acetic acid. The precipitant that developed during dialysis was also removed by centrifugation. The remaining supernatant was refolded as described (delCardayré et al., 1995). The removal of impurities by diluting the solubilized inclusion body with 20 mM acetic acid increased the yield of pure protein to 40–50 mg per 1 L of *E. coli* culture.

Preparation of the H12A RNase A·H119A RNase A dimer

A dimer of H12A RNase A and H119A RNase A was prepared as described elsewhere for the wild-type enzyme (Crestfield et al., 1962; Liu et al., 1998). The variants (2.7 mg of each) were mixed, dialyzed against distilled H_2O, and lyophilized. The lyophilized powder was dissolved in 50% (v/v) acetic acid (0.50 mL) to give a final concentration of 11 mg/mL, and incubated at 4 °C overnight. After the solution was lyophilized, the powder was dissolved in 0.50 mL of 0.20 M sodium phosphate buffer (pH 6.5) and loaded on a Pharmacia FPLC HiLoad 26/60 Superdex 75 gel filtration column, which had been equilibrated with 0.20 M sodium phosphate buffer (pH 6.5). Fractions corresponding to dimers were collected, and the concentration of dimer was determined spectroscopically by using $\epsilon = 0.72\ \text{mL mg}^{-1}\,\text{cm}^{-1}$ at 277.5 nm (Sela et al., 1957). The yield of dimers was ~20%, which is similar to a value reported recently (Liu et al., 1998).

Assays of ribonucleolytic activity

Ribonucleolytic activity was measured by monitoring cleavage of poly(C) with ultraviolet spectroscopy. The buffer was composed of 50 mM acetic acid, 50 mM MES, and 0.10 M Tris (Ellis & Morrison, 1982) containing NaCl (0.10 M). The pH was adjusted to pH 7.0 with aqueous HCl. After buffer (0.60 mL) containing poly(C) (0.042–0.83 mM) was equilibrated at 25 °C, the reaction was initiated by addition of enzyme. The increase in absorbance at 250 nm was monitored and used to calculate the reaction rate with $\Delta\epsilon = 2{,}380\ \text{M}^{-1}\,\text{cm}^{-1}$ (delCardayré & Raines, 1994). Kinetic parameters were determined by nonlinear regression analysis of kinetic data with the program SIGMAPLOT 5.0 (SPSS, Chicago, Illinois).

Assays of dimer dissociation

The dissociation of dimer was monitored by measuring the loss of ribonucleolytic activity. A solution of the H12A RNase A·H119A RNase A dimer (6 μM) in 0.20 M sodium phosphate buffer (pH 6.5) was heated to 37 or 65 °C. At known time intervals, small aliquots were removed and assayed for ribonucleolytic activity. When the dimer was incubated at 65 °C, the aliquots taken from the reaction were chilled on ice before assaying activity to suppress further reaction. Assays of ribonucleolytic activity were performed as described above, except that the concentration of poly(C) was 0.17 mM. Specific activities were calculated by dividing the observed rates by the concentration of enzyme monomer (0.20 μM) in the assay mixtures.

Assays of dimer formation at neutral pH

The formation of dimers was monitored by measuring the gain of ribonucleolytic activity. A mixture of H12A RNase A (6 μM) and H119A RNase A (6 μM) were incubated at 37 and 65 °C in 0.20 M sodium phosphate buffer (pH 6.5). Aliquots of the reaction were withdrawn at known time intervals and assayed as described above.

Determination of K_d of the dimer at neutral pH

The concentration of dimer in the mixture of H12A RNase A and H119A RNase A was determined from the ribonucleolytic activities measured in the three-component buffer (pH 7.0) containing NaCl (0.10 M) and poly(C) (0.17 mM). As a reference for 100% dimerization, the specific activity of the H12A RNase A·H119A RNase A dimer formed by lyophilization was determined in the same manner. Once the specific activity of a mixture of H12A RNase A and H119A RNase A was determined, the concentration of dimer was calculated with Equation 1; and the K_d of the dimer was calculated with Equation 2.

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References

Adinolfi S, Piccoli R, Sica F, Mazzarella L. 1996. BS-RNase tetramers: An example of domain-swapped oligomers. *FEBS Lett 398*:326–332.

Barnard EA. 1969. Biological function of pancreatic ribonuclease. *Nature 221*:340–344.

Bennett MJ, Schlunegger MP, Eisenberg D. 1995. 3D domain swapping: A mechanism for oligomer assembly. *Protein Sci 4*:2455–2468.

Burbaum JJ, Raines RT, Albery WJ, Knowles JR. 1989. Evolutionary optimization of the catalytic effectiveness of an enzyme. *Biochemistry 28*:9293–9305.

Cafaro V, Bracale A, Di Maro A, Sorrentino S, D'Alessio G, Di Donato A. 1998. New muteins of RNase A with enhanced antitumor action. *FEBS Lett 437*:149–152.

Cafaro V, De Lorenzo C, Piccoli R, Bracale A, Mastronicola MR, Di Donato A, D'Alessio G. 1995. The antitumor action of seminal ribonuclease and its quaternary conformations. *FEBS Lett 359*:31–34.

Carroll SF, Barbieri JT, Collier RJ. 1988. Diphtheria toxin: Purification and properties. *Methods Enzymol 165*:68–76.

Ciglic MI, Jackson PJ, Raillard SA, Haugg M, Jermann TM, Opitz JG, Trabesinger-Ruf N, Benner SA. 1998. Origin of dimeric structure in the ribonuclease superfamily. *Biochemistry 37*:4008–4022.

Crestfield AM, Stein WH, Moore S. 1962. On the aggregation of bovine pancreatic ribonuclease. *Arch Biochem Biophys Suppl 1*:217–222.

Crestfield AM, Stein WH, Moore S. 1963. Properties and conformation of the histidine residues at the active site of ribonuclease. *J Biol Chem 238*:2421–2428.

D'Alessio G. 1995. Oligomer evolution in action? *Nat Struct Biol 2*:11–13.

D'Alessio G. 1999a. Evolution of oligomeric proteins. The unusual case of a dimeric ribonuclease. *Eur J Biochem 266*:699–708.

D'Alessio G. 1999b. The evolutionary transition from monomeric to oligomeric proteins: Tools, the environment, hypotheses. *Prog Biophys Mol Biol 72*:271–298.

D'Alessio G, Di Donato A, Mazzarella L, Piccoli R. 1997. Seminal ribonuclease: The importance of diversity. In: D'Alessio G, Riordan J, ed. *Ribonuclease: Structures and function*. New York: Academic Press. pp 383–423.

delCardayré SB, Raines RT. 1994. Structural determinants of enzymatic processivity. *Biochemistry 33*:6032–6037.

delCardayré SB, Ribó M, Yokel EM, Quirk DJ, Rutter WJ, Raines RT. 1995. Engineering ribonuclease A: Production, purification, and characterization of wild-type enzyme and mutants at Gln11. *Protein Eng 8*:261–273.

Di Donato A, Cafaro V, D'Alessio G. 1994. Ribonuclease A can be transformed into a dimeric ribonuclease with antitumor activity. *J Biol Chem 269*:17394–17396.

Di Donato A, Cafaro V, Romeo I, D'Alessio G. 1995. Hints on the evolutionary design of a dimeric RNase with special bioactions. *Protein Sci 4*:1470–1477.

Ellis KJ, Morrison JF. 1982. Buffers of constant ionic strength for studying pH-dependent processes. *Methods Enzymol 87*:405–426.

Fisher BM, Ha J-H, Raines RT. 1998a. Coulombic forces in protein-RNA interactions: Binding and cleavage by ribonuclease A and variants at Lys7, Arg10 and Lys66. *Biochemistry 37*:12121–12132.

Fisher BM, Schultz LW, Raines RT. 1998b. Coloumbic effects of remote subsites on the active site of ribonuclease A. *Biochemistry 37*:17386–17401.

Gotte G, Libonati M. 1998. Two different forms of aggregated dimer of ribonuclease A. *Biochim Biophys Acta 1386*:106–112.

Gould SJ. 1977. *Ever since Darwin: Reflections in natural history*. New York: W.W. Norton & Company. pp 103–110.

Graziano G, Catanzano F, Giancola C, Barone G. 1996. DSC study of the thermal stability of S-protein and S-peptide/S-protein. *Biochemistry 35*:13386–13392.

Grimsley GR, Shaw KL, Fee LR, Alston RW, Huyghues-Despointes BM, Thurlkill RL, Scholtz JM, Pace CN. 1999. Increasing protein stability by altering long-range coulombic interactions. *Protein Sci 8*:1843–1849.

Harber E, Anfinsen CA. 1961. Regeneration of enzyme activity by air oxidation of reduced subtilisin-modified ribonuclease. *J Biol Chem 236*:422–426.

Highbarger LA, Gerlt JA, Kenyon GL. 1996. Mechanism of the reaction catalyzed by acetoacetate decarboxylase. Importance of lysine 116 in determining the pK_a of active-site lysine 115. *Biochemistry 35*:41–46.

Hummel CF, Pincus MR, Brandt-Rauf PW, Frei GM, Carty RP. 1987. Reaction of (bromoacetamido)nucleoside affinity labels with ribonuclease A: Evidence for steric control of reaction specificity and alkylation rate. *Biochemistry 26*:135–146.

Kao CM, Pieper R, Cane DE, Khosla C. 1996. Evidence for two catalytically independent clusters of active sites in a functional modular polyketide synthase. *Biochemistry 35*:12363–12368.

Kim J-S, Soucek J, Matousek J, Raines RT. 1995. Structural basis for the biological activities of bovine seminal ribonuclease. *J Biol Chem 270*:10525–10530.

Kraulis PJ. 1991. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J Appl Crystallogr 24*:946–950.

Larimer FW, Lee EH, Mural RJ, Soper TS, Hartman FC. 1987. Intersubunit location of the active site of ribulose-bisphosphate carboxylase/oxygenase as determined by in vivo hybridization of site-directed mutants. *J Biol Chem 262*:15327–15329.

Li Y, Feng L, Kirsch JF. 1997. Kinetic and spectroscopic investigations of wild-type and mutant forms of apple 1-aminocyclopropane-1-carboxylate synthase. *Biochemistry 36*:15477–15488.

Liu Y, Hart PJ, Schlunegger MP, Eisenberg D. 1998. The crystal structure of a 3D domain-swapped dimer of RNase A at a 2.1 Å resolution. *Proc Natl Acad Sci USA 95*:3437–3442.

Loladze VV, Ibarra-Molero B, Sanchez-Ruiz JM, Makhatadze GI. 1999. Engineering a thermostable protein via optimization of charge–charge interactions on the protein surface. *Biochemistry 38*:16419–16423.

Mazzarella L, Capasso S, Demasi D, Di Lorenzo G, Mattia CA, Zagari A. 1993. Bovine seminal ribonuclease: Structure at 1.9 Å resolution. *Acta Crystallogr D 49*:389–402.

Messmore JM, Holmgren SK, Grilley JE, Raines RT. 2000. The sulfur shuffle: Modulating enzymatic activity by thiol–disulfide interchange. *Bioconjug Chem 11*:408–413.

Perl D, Mueller U, Heinemann U, Schmid FX. 2000. Two exposed amino acid residues confer thermostability on a cold shock protein. *Nat Struct Biol 7*:380–383.

Piccoli R, Di Gaetano S, De Lorenzo C, Grauso M, Monaco C, Spalletti-Cernia D, Laccetti P, Cinatl J, Matousek J, 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.

Piccoli R, Tamburrini M, Piccialli G, Di Donato A, Parente A, D'Alessio G. 1992. The dual-mode quaternary structure of seminal RNase. *Proc Natl Acad Sci USA 89*:1870–1874.

Quirk DJ, Park C, Thompson JE, Raines RT. 1998. His · · · Asp catalytic dyad of ribonuclease A: Conformational stability of the wild-type, D121N, D121A, and H119A enzymes. *Biochemistry 37*:17958–17964.

Richards FM. 1955. Titration of amino groups released during the digestion of ribonuclease by subtilisin. *C R Trav Lab Carlsberg Ser Chim 29*:322–328.

Richards FM, Vithayathil PJ. 1959. The preparation of subtilisin-modified ribonuclease and the separation of the peptide and protein components. *J Biol Chem 234*:1459–1465.

Russo N, Antignani A, D'Alessio G. 2000. In vitro evolution of a dimeric variant of human pancreatic ribonuclease. *Biochemistry 39*:3585–3591.

Russo N, Shapiro R. 1999. Potent inhibition of mammalian ribonucleases by 3′,5′-pyrophosphate-linked nucleotides. *J Biol Chem 274*:14902–14908.

Schlunegger MP, Bennett MJ, Eisenberg D. 1997. Oligomer formation by 3D domain swapping: A model for protein assembly and misassembly. *Adv Protein Chem 50*:61–122.

Sela M, Anfinsen CB, Harrington WF. 1957. The correlation of ribonuclease activity with specific aspects of tertiary structure. *Biochim Biophys Acta 26*:502–512.

Shoemaker KR, Fairman R, Kim PS, York EJ, Stewart JM, Baldwin RL. 1987. The C-peptide helix from ribonuclease A considered as an autonomous folding unit. *Cold Spring Harbor Symp Quant Biol 52*:391–398.

Sorrentino S, Barone R, Bucci E, Gotte G, Russo N, Libonati M, D'Alessio G. 2000. The two dimeric forms of RNase A. *FEBS Lett 466*:35–39.

Spector S, Wang M, Carp SA, Robblee J, Hendsch ZS, Fairman R, Tidor B, Raleigh DP. 2000. Rational modification of protein stability by the mutation of charged surface residues. *Biochemistry 39*:872–879.

Stowell JK, Widlanski TS, Kutateladze TG, Raines RT. 1995. Mechanism-based inactivation of ribonuclease A. *J Org Chem 60*:6930–6936.

Thompson JE, Raines RT. 1995. Value of general acid–base catalysis to ribonuclease A. *J Am Chem Soc 116*:5467–5467.

Tobias KE, Kahana C. 1993. Intersubunit location of the active site of mammalian ornithine decarboxylase as determined by hybridization of site-directed mutants. *Biochemistry 32*:5842–5847.

van Gent DC, Vink C, Groeneger AA, Plasterk RH. 1993. Complementation between HIV integrase proteins mutated in different domains. *EMBO J 12*:3261–3267.

Warshel A. 1998. Electrostatic origin of the catalytic power of enzymes and the role of preorganized active sites. *J Biol Chem 273*:27035–27038.

Wente SR, Schachman HK. 1987. Shared active sites in oligomeric enzymes: Model studies with defective mutants of aspartate transcarbamoylase produced by site-directed mutagenesis. *Proc Natl Acad Sci USA 84*:31–35.

Witkowski A, Joshi A, Smith S. 1996. Fatty acid synthase: *In vitro* complementation of inactive mutants. *Biochemistry 35*:10569–10575.