In Vitro Evolution of a Dimeric Variant of Human Pancreatic Ribonuclease†

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ABSTRACT: Site-directed mutagenesis of human pancreatic RNase (HP-RNase) was used as a model system for investigating the genetic events underlying the evolutionary origins of protein oligomers. HP-RNase is a monomeric enzyme with no natural tendency to oligomerize (K_d for any dimers in solution of >280 mM). Nevertheless, deletion of five amino acid residues in the loop linking the N-terminal helix of HP-RNase to the rest of the protein was found to drive polypeptide chains to fold into dimers. These dimers could not be dissociated by heating at 70 °C, and small amounts of monomer were detected only in highly diluted samples. Measurement of dimer and monomer concentrations under equilibrium conditions yielded a K_d of 1.5 μM. This implies that the deletion increases the protein propensity to dimerize at least 5.2 orders of magnitude. Moreover, the HP-RNase dimers were found to be over 4.6 orders of magnitude more stable than the dimers of bovine pancreatic RNase A obtained by lyophilization from acetic acid (K_d > 73 mM). Cross-linking experiments with divinyl sulfone indicated that the HP-RNase dimers are stabilized by the exchange between subunits of their N-terminal helices. This generates composite active sites, i.e., each contributed by two subunit chains, that retain full enzymatic activity. Overall, these results show that a deletion of few residues in a key region of a monomeric protein can be the primary event irreversibly leading to oligomerization of the protein through the swap of a secondary structure element between protomers.

An increasing attention has been given in recent years to the interesting question of the evolutionary origins of protein oligomers (*1*−*4*). In this respect, a particular interest has been shown for a versatile model system made up of two well-characterized members from the superfamily of pancreatic-type RNases (*1*, *2*, *5*−*7*). One member is monomeric RNase A[1] from bovine pancreas (*8*), the well-known superfamily prototype (*9*); the other is dimeric BS-RNase from bovine seminal plasma (see ref *10* for a review). The system is enriched by the availability of artificial dimers of RNase A, prepared by lyophilization of highly concentrated protein samples from 50% acetic acid. Such acidic denaturing conditions and the increase of protein concentration occurring during the lyophilization have been proposed as responsible for the aggregation process (*11*), which involves only a fraction of the RNase molecules (about 20%). Recently, the three-dimensional structure of RNase A dimers has been elucidated (*12*). It shows that the two RNase A subunits associate through the interchange or swap (*13*) of their N-terminal helices, as in the case of naturally dimeric BS-RNase (*14*). The monomeric/dimeric RNase pair has in fact been considered as one of the cases that illustrate the "3D domain-swapping model" (*1*) of oligomer evolution. This model does not require a lengthy accumulation of mutations to build an adhesive surface for monomer association, as the swapped domain or structural element just recreates in the dimer an interface already present in the monomers. The swapping of structural parts between subunits has also been reported in other naturally occurring dimeric proteins, such as bovine odorant binding protein and β-crystallins (*13*).

A key element which still needs to be deciphered for describing a case of oligomer evolution according to the "swap model" is that of the genetic event priming the swap. If the swappable domain reproduced in the dimer exactly the same interactions that it forms in a monomer, then there would not be any enthalpic factors contributing to dimer formation. On the contrary, there would be an unfavorable entropic pressure from the loss of monomer freedom that should ultimately hinder association. In this regard, it should be noted that the RNase A dimers, the products of an artificial dimerization process that does not involve mutations, are not stable. They spontaneusly and irreversibly dissociate to monomers at a rate that is dependent on the temperature (*15*). In the case of BS-RNase, instead, dimers are very stable, but much of their stability is apparently due to the presence of two intersubunit disulfide bridges. Under this scenario, it

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[1] Abbreviations: RNase A, bovine pancreatic RNase A; BS-RNase, bovine seminal RNase; HP-RNase, human pancreatic RNase; des(16−18)HP and des(16−20)HP, deletion mutants of HP-RNase that miss the residues indicated in parentheses; PCR, polymerase chain reaction; FPLC, fast-protein liquid chromatography; SDS−PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

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is apparent that a swapping event leading to stable dimers must be generated by mutation(s) in critical regions of the protein structure.

The identification of this "primary mutation" (*4*), the mutational event that primed the dimerization of the monomeric protein, would require the knowledge of the amino acid sequence of the ancestral monomer and that of the newly generated dimer, whereas only present day monomer and dimer can be analyzed. However, it has been predicted (*1*, *4*) that mutations in the sequence encoding the surface loop that links a domain or structural element to the rest of a monomeric protein can induce the swap of the domain or structural element between protomers, hence oligomerization. In fact, a dimeric variant of staphylococcal nuclease was unexpectedly recovered (*16*) when six amino acid residues were deleted in a surface loop connecting the C-terminal helix to the main body of the monomer. Furthermore, a high mutation rate has been determined (*6*) for the sequence encoding the surface peptide segment that links in RNases the main protein body with the N-terminal helix. This segment is hinge in the exchange of the N-terminal helix between protomers in dimeric RNase A and BS-RNase.

In this study, we investigated the genetic events priming the swap by considering the effects of deletions on a monomeric ribonuclease, human pancreatic RNase (HP-RNase; refs *17* and *18*). The choice of this enzyme as a model protein was motivated by (i) the availability of a cDNA encoding the protein (*19*) and of an *Escherichia coli* system for RNase expression (*20*); (ii) the RNase polypeptide fold and architecture of the active site (*21*), quite different from those of staphylococcal nuclease (*22*); and (iii) preliminary results showing that HP-RNase has no natural tendency to dimerize, even when the enzyme is lyophilized from 50% acetic acid.

Deletions were made in the DNA segment encoding the polypeptide loop that links the N-terminal helix of HP-RNase to the main protein body. We found that these deletions are indeed able to promote swapping of the N-terminal helix between subunits and to produce very stable dimers.

EXPERIMENTAL PROCEDURES

Materials and General Procedures. Bacterial cultures, restriction endonuclease digestions, DNA ligations, and bacterial transformations were performed according to Sambrook et al. (*23*). Plasmids were purified with a Qiaprep Spin Miniprep kit (Qiagen). Double-stranded DNA was sequenced with the dideoxynucleotide-termination method of Sanger using a T7 Sequenase 2.0 Sequencing kit (Amersham). SDS−PAGE analyses were performed according to Laemmli (*24*), using 15% polyacrylamide gels that were stained with Coomassie blue. Protein sequence analyses were performed with an Applied Biosystems sequencer model 473A, equipped with a high-pressure liquid chromatography apparatus for identification of phenylthiohydantoin derivatives. Dimeric RNase A was kindly provided by Dr. Salvatore Sorrentino of this Department. Ribonucleolytic activity toward yeast RNA was measured according to Kunitz (*25*), except that 0.15 M NaCl was included in the assay buffer to increase enzyme solubility.

Site-Directed Mutagenesis. Mutant cDNAs coding for des(16−18)HP and des(16−20)HP were prepared by the PCR-based overlap extension method of Ho et al. (*26*). The template for PCR was the expression plasmid pET-HP, kindly provided by Dr. Valeria Cafaro of this department. This plasmid is composed of a synthetic cDNA coding for wild-type HP-RNase (*19*), ligated to *Nde*I and *Hin*dIII restriction sites of the *E. coli* expression vector pET-22b (Novagen). The cDNA sequence is identical to that previously reported (nucleotides 98-481 in ref *27*) except for a silent mutation (C133T) introduced to delete an internal *Nde*I site. PCR mixtures were set up using a GeneAmp kit (Perkin-Elmer) and contained various oligonucleotides. Mutagenic primers for preparing des(16−18)HP cDNA were oligonucleotides 5′-CATGGACTCTCCGTCTTCTTCTTCTACGTA-3′ and 5′-AAGAAGACGGAGAGTCCATGTGTTGACGTT-3′; those for des(16−20)HP cDNA were oligonucleotides 5′-CATGGACTCTTCTTCTTCTACGTACTGCAA-3′ and 5′-TAGAAGAAGAAGAGTCCATGTGTTGACGTT-3′; the outmost amplification primers were T7 promoter and T7 terminator oligonucleotides (Novagen).

DNA amplifications were performed by 30 thermal cycles each composed of a 2-min denaturation step at 95 °C, a 2-min annealing step at 50 °C, and a 2-min extension step at 72 °C. PCR products were gel-purified with a Wizard PCR Preps kit (Promega), digested with *Nde*I and *Hin*dIII, and ligated into pET-22b that had been cleaved with the same enzymes. JM101 cells were then transformed with the resulting plasmids. Finally, the PCR-derived region of each plasmid was sequenced to establish the presence of the programmed mutations and rule out any spurious changes.

Protein Expression and Purification. The expression plasmid pET-HP, containing the cDNA coding for wild-type HP-RNase, and its two derivatives for the espression of des(16−18)HP and des(16−20)HP were separately used to transform cells of the *E. coli* strain BL21(DE3) (Novagen). Transformed cells were then cultured in 0.5 l of Terrific Broth medium (*23*) containing 50 μg/mL ampicillin. When the absorbance of the culture at 600 nm reached a value of three, cells were induced with 0.4 mM isopropyl β-D-thiogalactopyranoside for 16 h. Bacteria were then harvested by centrifugation at 5500*g* for 15 min, resuspended in 40 mL of lysis buffer (0.1 M Tris-Cl, pH 8.0, containing 5 mM EDTA) and treated with 0.2 mg/mL lysozyme for 1 h at room temperature on a rocking platform. Cells were disrupted by sonication, and crude inclusion bodies, containing the expressed proteins, were collected by centrifugation at 17000*g* for 30 min. They were then washed four times with 30 mL of lysis buffer containing 2% Triton X-100 and 2 M urea and twice with 30 mL of lysis buffer alone.

Expressed proteins were then extracted from the inclusion bodies, refolded, and purified, following a procedure successfully used for other RNases (*20*). This procedure yielded reasonable amounts of wild-type HP-RNase, whereas the two deletion mutants were obtained in very little amounts. The low yields of the two HP-RNase mutants could then be attributed to proteolytic cleavages of their polypeptide chains, occurring during the refolding step. It may not be surprising that a contaminant protease did not have instead any effects on the wild-type protein. Apparently this protease, present in the reoxidation buffer, would refold more slowly than the wild-type HP-RNase, but more quickly than the two mutant proteins. This proteolysis problem was later solved by performing the refolding at low temperature and in the

presence of protease inhibitors, as detailed below.

Unless otherwise specified, the following steps were performed at 4 °C. Inclusion bodies containing unfolded wild-type HP-RNase, des(16−18)HP, or des(16−20)HP were suspended in 10 mL of 50 mM Tris-Cl, pH 7.0, containing 10 mM EDTA, 6 M guanidine-Cl, 0.57 mM phenylmethanesulfonyl fluoride, and 92 mM glutathione and stirred for 90 min. Samples were then diluted with 190 mL of reoxidation buffer (0.1 M Tris-Cl, pH 8.4, containing 10 mM EDTA, 0.5 M L-arginine, 0.28 mM phenylmethanesulfonyl fluoride, and 1 mM oxidized glutathione), saturated with N_2, and let stand for 23 h. The concentrations of the three recombinant proteins in the reoxidation mixtures were between 50 and 100 μg/mL. Refolded protein samples were then clarified by centrifugation at 12000*g* for 30 min and dialyzed extensively against 10 mM Tris-Cl, pH 6.8. This treatment was used to precipitate HP-RNase and its mutants, as they are poorly soluble at low ionic strength (*17*). Precipitates were collected by centrifugation as above, suspended in 13 mL of 0.1 M Tris-Ac, pH 7.0, containing 0.3 M NaCl and 0.57 mM phenylmethanesulfonyl fluoride, and stirred for 16 h. Samples were then clarified by centrifugation performed as above and finally fractionated by gel filtration on a FPLC apparatus (Pharmacia) equipped with a Superdex 75 26/60 column, previously equilibrated with 0.1 M Tris-Ac, pH 7.0, containing 0.3 M NaCl. Chromatography was performed at room temperature at a flow rate of 2.5 mL/min. RNase fractions were pooled and stored at −20 °C.

Stock concentrations of HP-RNase and its mutants were determined by a colorimetric assay (BCA kit, Pierce), with reference to a calibration curve obtained with known amounts of RNase A. Final yields of homogeneous HP-RNase, des(16−18)HP, and des(16−20)HP were between 5 and 9 mg/liter of bacterial culture.

RNase Reaction with Divinyl Sulfone. HP-RNase, des(16−20)HP, and BS-RNase were first equilibrated in 0.1 M Na-Ac, pH 5.0, containing 0.2 M NaCl, by an extensive dialysis at 4 °C. Then, 14 μg of each RNase was diluted in 100 μL of the same buffer, and treated with divinyl sulfone (Sigma) for 48 h as described (*7*). Finally, 10 μL of the resulting samples was analyzed by SDS−PAGE under reducing conditions.

Dimer Dissociation Kinetics. Aliquots of des(16−20)HP (17 μg) were diluted in 0.1 M Tris-Ac, pH 7.0, containing 0.3 M NaCl, to final protein concentrations of 3−23 μM. Samples were then incubated at 37 or 70 °C. After suitable time intervals, each sample was cooled to room temperature and immediately chromatographed by gel filtration/FPLC on a Superdex 75 10/30 column (Pharmacia) equilibrated with the same buffer, at a flow rate of 0.6 mL/min. The amounts of monomer and dimer were then estimated by measuring the areas of their profiles of absorbance at 278 nm. Reported value of dissociation constant of des(16−20)HP dimers is the mean ± SD. Lower limits of dissociation constants of dimeric RNase A and of any dimeric forms of wild-type HP-RNase were determined at 37 °C under the same buffer conditions as reported above.

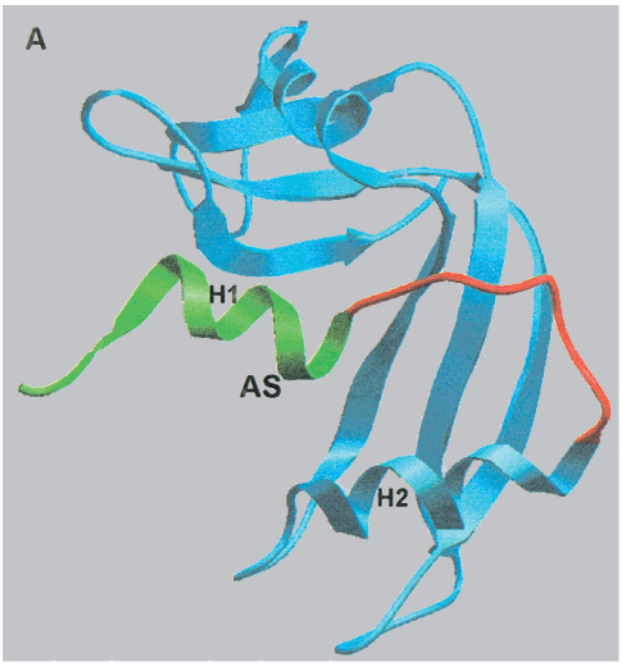
Molecular Modeling. The structural model for dimeric des(16−20)HP was prepared with the aid of SWISS-MODEL (*28*), a freely accessible server for automated comparative protein modeling (http://www.expasy.ch/swissmod/SWISS-MODEL.html). Specifically, the program ProModII (*29*) was used to build two models for monomeric des(16−20)HP, based on the atomic coordinates of the two subunits of the RNase A dimer (Protein Data Bank code 1A2W; ref *12*). The two monomeric models were then combined and energy minimized with Gromos96 (*28*). The resulting dimer model was finally examined with the program SWISS−PdbViewer (*30*).

RESULTS AND DISCUSSION

Design and Preparation of Two Dimeric Variants of Human Pancreatic RNase. The 3D structure of HP-RNase has yet to be reported. However, its high degree of sequence identity with RNase A (70%; refs *8* and *18*) suggests that the two proteins have a very similar folding. This assumption is supported by the previous finding that another pancreatic-type RNase, eosinophil-derived neurotoxin, exhibiting only 36% sequence identity with RNase A, shares its folding with a root-mean-square deviation for the position of homologous α-carbons of only 1.27 Å (loops excluded; ref *31*). Finally, a recent nuclear magnetic resonance study of the secondary structure of HP-RNase has shown that its N-terminal region, on which this study is focused, is indeed structured almost identically to that of RNase A (*32*). The design of HP-RNase mutants could hence be based on the polypeptide fold of RNase A.

RNase A is a globular protein formed by two β-sheets surrounded by three α-helices (Figure 1A). Helix-1 extends in the N-terminal region (residues 3−13) and contributes to the active site the substrate-binding residues Lys-7 and Arg-10 and the catalytic residue His-12. The two other catalytic residues, Lys-41 and His-119, are on the main protein body, which is stabilized by four disulfide bridges and connected to helix-1 by a peptide loop comprising residues 14−23. This loop has been shown to be flexible in RNases (*33*), hence potentially able to accommodate small structural changes. On the basis of this key factor and on the results reported in the introductory portion of this paper, it was thought that shortening of the loop 14−23 of HP-RNase may preclude helix-1 from folding in its natural position on the main protein body. Deletions in this loop should then destabilize the protein and eventually lead to the folding of helix-1 onto the main body of an oncoming protomer. This would generate swapped dimers, with composite active sites, i.e. each formed by two polypeptide chains. Two deletion mutants were planned: des(16−18)HP and des(16−20)HP, with the loop 14−23 reduced by 30% (three residues deleted) and 50% (five residues deleted), respectively (Figure 1B).

Mutant cDNAs coding for des(16−18)HP and des(16−20)HP were prepared by PCR and expressed in *E. coli*. The recombinant proteins were then recovered from inclusion bodies, refolded, and purified by gel filtration. As a control, wild-type HP-RNase was expressed, refolded, and purified under exactly the same conditions employed for the two mutants. This control was performed to ensure that the expression system did not yield any artifactual dimers of the wild-type protein. Gel-filtration elution profiles (Figure 2) show that HP-RNase behaves as a monomeric protein, whereas des(16−18)HP is eluted as a mixture of dimer and monomer in an approximate ratio of 4:1, and des(16−20)HP appears to be entirely dimeric. No higher oligomers could be detected in any of the three protein samples.



B

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                 <   H1    ><   L    >< H2
RNase A          KETAAAKFERQHMDSSTSAASSSNYCN
HP-RNase         KESRAKKFQRQHMDSDSSPSSSSTYCN
Des(16-18)HP     KESRAKKFQRQHMDS---PSSSSTYCN
Des(16-20)HP     KESRAKKFQRQHMDS-----SSSTYCN
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FIGURE 1: (A) Polypeptide fold of RNase A showing the loop 14−23 (red) connecting the N-terminal helix (H1, green) to helix-2 (H2) that belongs to the main protein body (cyan blue). The active site (AS) is on the back of helix-1 and comprises residues from both this helix and the rest of the protein. (B) Sequence alignment of the N-terminal regions of RNase A, HP-RNase, and the two deletion mutants considered in this study, des(16−18)HP and des(16−20)-HP. Panel A was prepared using the program SWISS-PdbViewer (*30*) and the atomic coordinates of Wlodawer et al. (Protein Data Bank code 7RSA; ref *21*).

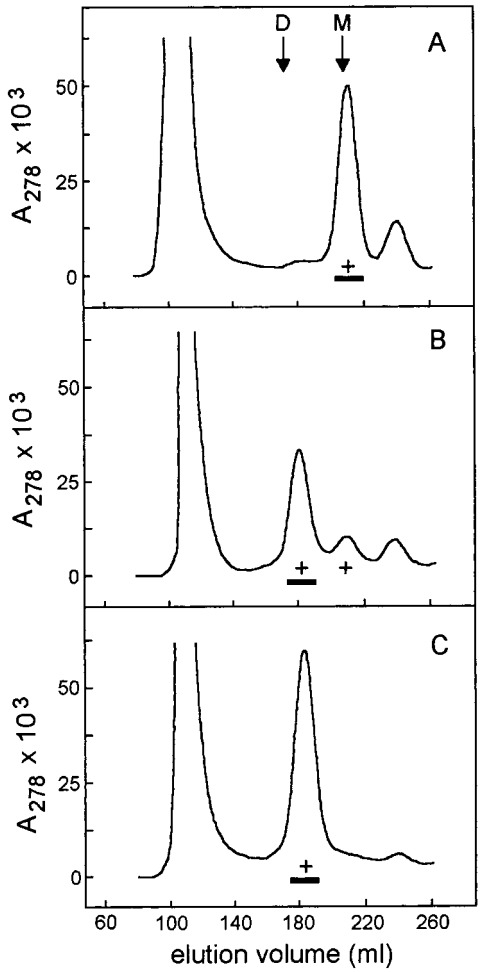

FIGURE 2: Gel-filtration profiles of crude samples of recombinant HP-RNase (A), des(16−18)HP (B) and des(16−20)HP (C). M and D indicate the elution volumes of a monomeric RNase (RNase A; M_r = 13 700 Da) and a dimeric RNase (BS-RNase; M_r = 27 400 Da), respectively. The plus sign marks the RNase-containing peaks. Bars indicate the RNase fractions used for further characterization.

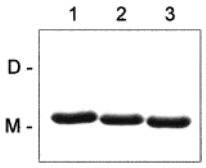

FIGURE 3: SDS−PAGE under nonreducing conditions of HP-RNase and its dimeric variants. Lanes 1−3: 4 μg of HP-RNase, des(16−18)HP, and des(16−20)HP, respectively. M and D mark the migrations of a monomeric and a dimeric RNase, respectively, as detailed in Figure 2.

These data indicate that deletions in the loop 14−23 of HP-RNase are indeed capable of promoting protein dimerization and that a deletion of five residues is more effective than one of three residues.

The chromatographic fractions of monomeric HP-RNase and those of the two dimeric variants were pooled and used for further characterization.

Structural and Functional Characterization of HP-RNase Dimers. N-Terminal sequencing of the first 20 amino acid residues of the two HP-RNase mutants, des(16−18)HP and des(16−20)HP, provided direct evidence of the introduced mutations.

The HP-RNase dimers were then analyzed by SDS−PAGE under nonreducing conditions. Their electrophoretic migration was found to be slightly higher than that of wild-type HP-RNase (Figure 3), as expected for single polypeptide chains with deletions of few residues. On one hand, this result provides further evidence of the introduced mutations, on the other, it demonstrates that the two dimers are stabilized only by noncovalent interactions. If the two dimers were misfolded proteins with intersubunit disulfides, they would, in fact, migrate as dimers under nonreducing electrophoresis conditions.

The two dimeric variants of HP-RNase were then tested for their ability to digest yeast RNA, a conventional RNase substrate. The specific activity of des(16−18)HP and des-(16−20)HP resulted to be 20 ± 5 and 22 ± 1 units/mg, respectively. These values are virtually identical to that measured, under the same assay conditions, for wild-type HP-RNase (21 ± 5 units/mg). These data suggest that the two ribonucleolytic centers of each dimer are structured canonically, with the active-site residues of helix-1, Lys-7, Arg-10, and His-12, properly positioned. This can be interpreted by two hypotheses: (i) a swapping event has occurred, and each helix-1-folds onto the main body of an adjacent protomer, or (ii) there is no swapping, and the two subunits are maintained by some other interactions.

To verify these hypotheses, the HP-RNase mutant with a higher propensity to dimerize, des(16−20)HP, was reacted

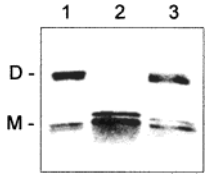

FIGURE 4: Swapping test. BS-RNase, HP-RNase, and des(16−20)-HP were treated with divinyl sulfone and analyzed by SDS−PAGE under reducing conditions, lanes 1−3, respectively. M and D mark the migrations of a monomeric and a dimeric RNase, respectively, as detailed in Figure 2. Dual bands arise from the divinyl sulfone-induced cleavage of the RNase polypeptide chain near its C-terminus (*8*).

with a bifunctional reagent, divinyl sulfone, previously used to assess the swap of helix-1 in BS-RNase and in the artificial dimers of RNase A (*7*), as it reacts with the catalytical residues His-12 and His-119. When the active site of a dimer is composite, with His-12 from one subunit and His-119 from the other, the cross-link joins the two subunits, yielding a covalent dimer. If the active site of a dimer is not composite, divinyl sulfone acts as it does in a monomeric RNase, linking two histidines from the same polypeptide chain. Reaction with divinyl sulfone was also performed with two control proteins, monomeric HP-RNase, and BS-RNase, a swapped dimer (*14*). SDS−PAGE was then used to distinguish covalent dimers from noncovalent ones or from monomers. Figure 4 shows that reacted HP-RNase migrates as a monomer, whereas des(16−20)HP and BS-RNase migrate, after the reaction, as dimers. This result demonstrates that the quaternary structure of des(16−20)HP is stabilized by the swapping of the N-terminal helices.

Stability of Dimeric des(16−20)HP RNase. Thermal stability was first considered. RNase A dimers are known to be metastable: when heated at 65 °C, a temperature above the melting temperature of RNase A, they rapidly dissociate into monomers and, upon cooling, they do not reassociate into dimers (*15*). To assess whether des(16−20)HP was metastable, too, a 23 μM solution was heated at 70 °C for 5 min, cooled to room temperature, and analyzed by gel filtration to reveal any resulting monomeric species. The elution profile (Figure 5A) showed that the heated protein is still almost entirely dimeric. This result indicates that des-(16−20)HP dimers are very stable: they may be able to resist thermal dissociation, or more likely, they are dissociated by heating but refold into dimers upon cooling. If the latter hypothesis is valid, protein concentration may affect the reassociation process, yielding less dimer at lower protein concentrations in the heating−cooling experiment. A diluted sample of des(16−20)HP (5 μM) was thus heated-cooled as above and analyzed. In this case, gel filtration revealed a more substantial amount of monomer (Figure 5B). This strongly suggests that dimers are dissociated by heating and that the resulting monomers then refold into dimers to an extent that depends on protein concentration.

Dimer stability was then investigated at a physiological temperature. A diluted sample of des(16−20)HP at 5 μM concentration was incubated at 37 °C for 24 h and analyzed. Again, small amounts of monomer were detected. Incubations at 37 °C for various times then showed that dissociation is time dependent, with a $t_{1/2}$ of 3 h (data not shown). The dissociation constant of des(16−20)HP was then determined by measuring the amounts of monomer and dimer under

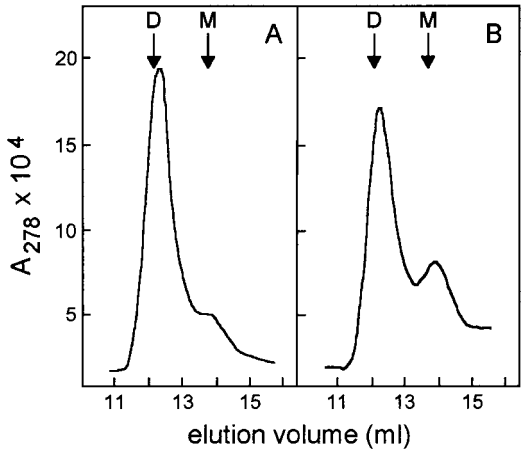

FIGURE 5: Effect of heating and protein concentration on the dissociation of des(16−20)HP. Aliquots of des(16−20)HP at concentrations of 23 μM (A) and 5 μM (B) were heated at 70 °C for 5 min, cooled to room temperature, and analyzed by gel filtration. M and D mark the elution volumes of a monomeric and a dimeric RNase, respectively, as detailed in Figure 2.

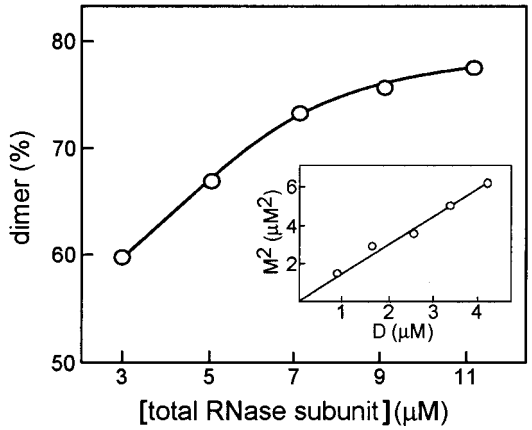

FIGURE 6: Measurement of the dissociation constant of des(16−20)HP. Samples of des(16−20)HP at 3−11 μM concentrations were equilibrated at 37 °C for 24 h and analyzed by gel filtration to measure the fractions of monomer and dimer. The main plot shows the amounts of dimer at various protein concentrations. The insert shows a linearization of the data, where M and D indicate monomer and dimer concentrations, respectively. In this plot, K_d is given by the slope ($[M]^2/[D]$).

equilibrium conditions at various protein concentrations. The protein was diluted to 3−11 μM, equilibrated for 24 h at 37 °C, and then analyzed. As shown in Figure 6, the amount of dimer increases with protein concentration. It is remarkable that at concentrations as low as 3 μM, 60% of the protein is still dimeric. Linearization of the data (see inset of Figure 6) yielded a K_d of 1.54 ± 0.17 μM. As a control, a sample of wild-type HP-RNase was concentrated to 140 μM, incubated at 37 °C for 7 days, and analyzed by gel filtration to reveal any dimer formation. As a result, no dimeric or oligomeric species could be detected (detection limit = 70 nM dimer). This allowed an estimate of the lower limit of dissociation constant for any dimers of HP-RNase in solution as K_d > 280 mM. These data indicate that the monomeric form of des(16−20)HP has a propensity to dimerize at least 5.2 orders of magnitude higher than that of the wild-type enzyme. An attempt was also made to measure the K_d of the RNase A dimers. A 36 μM dimer sample was equilibrated at 37 °C for 7 days and analyzed by gel filtration. This revealed that dimers were completely dissociated to mono-

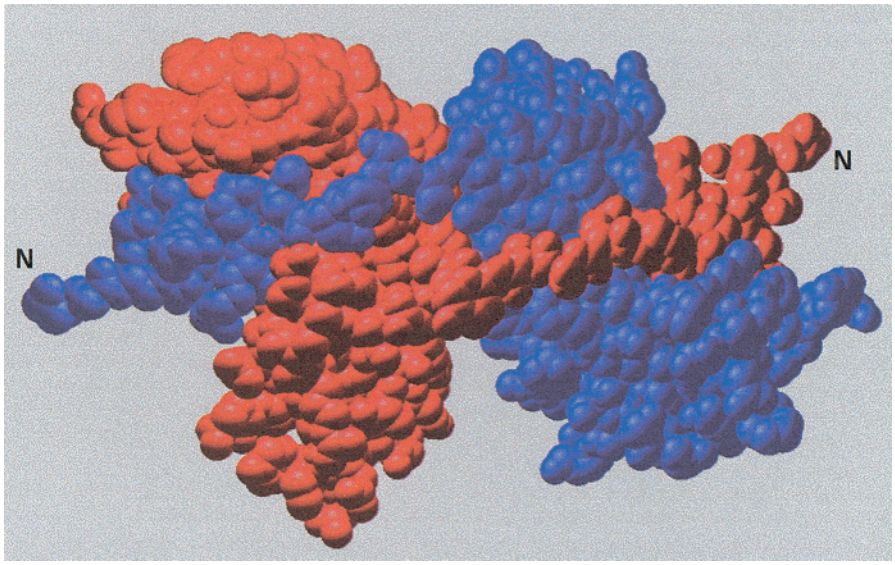

FIGURE 7: Structural model for des(16−20)HP dimer. Each atom is represented by its van der Waals surface. The two RNase subunits are colored in red and blue. The red subunit is oriented as in Figure 1, except for the swapped helix-1. N marks the N-terminal end of each polypeptide chain. The figure was prepared with the program SWISS-PdbViewer (*30*).

mers and allowed an estimate of a K_d value higher than 73 mM. This indicates that des(16−20)HP dimers are at least 4.6 orders of magnitude more stable than those of RNase A.

Molecular Model for Dimeric des(16−20)HP RNase. Molecular modeling was used to investigate the quaternary structure of des(16−20)HP. Comparative protein modeling was employed to build two dimeric models based on the crystal structures of two different types of dimers, those of RNase A (*12*) and BS-RNase (*14*). In both dimers, the swapping between subunits of their N-terminal helices generates a primary interface characterized by the same interactions that are observed within monomeric RNases. This interface is defined as "closed" (*13*), since it is not exposed to the solvent in a monomer. However, the relationships of the subunits in the two dimers are quite different. In BS-RNase dimer, a secondary "open" interface (water exposed in monomeric RNases) is formed through the interactions of the helices-2 of the two subunits, and a dimer results with a 2-fold molecular symmetry. In contrast, the two subunits of the RNase A dimer are arranged asymmetrically, related by a rotation of 160°. In this case, an "open" interface is formed between two antiparallel β-strands of the two protomers.

The model based on BS-RNase failed to fit the experimental data, as the five-residue deletion in the loop 14−23 could not be accommodated without seriously altering the architecture of the active sites (data not shown). On the other hand, the model based on RNase A dimer appeared realistic (Figure 7). The deletion could be accommodated in both subunits, in part by a more extended conformation of the residual peptide loop and, in part, by reducing the extension of helix-2. Helix-1 appeared not to be altered and could be positioned at the active site exactly as in a monomeric RNase. The remaining part of the model resembled very closely the structure of the reference protein, except for a low degree of molecular complementarity between the two β-strands that form the secondary interface. In conclusion, the preceding exercise suggested that des(16−20)HP cannot form BS-RNase-like dimers but can fold into RNase A-like dimers.

Conclusions and Implications. A single mutation, deletion of five amino acid residues, is here found to be able to transform a monomeric RNase into a stably dimeric enzyme. The dimeric structure is achieved through the interchange between subunits of a secondary structure element with subsequent formation of composite active sites that retain full enzymatic activity. It is remarkable that two out of two deletion mutants considered in this study are able to fold, at least partially, into dimers.

This is in our knowledge only the second example of swapping of structural elements, and subsequent protein dimerization, achieved by site-directed mutagenesis. The first one relates to the serendipitous finding of a dimeric mutant of staphylococcal nuclease characterized by the swapping of the C-terminal helices (*16*). As the latter protein displays a polypeptide fold and an architecture of the active site quite different from those of pancreatic-type ribonucleases (*21*, *22*), the results reported here show that deletions in a loop connecting a domain or structural element with the rest of a monomeric protein may be a more general genetic mechanism for priming the swap of parts in monomeric proteins and for inducing their dimerization. The effect of deletions on the oligomerization state may be interpreted by assuming that they are able to destabilize the monomer so that a secondary structure element can dissociate from it and fold on an oncoming protomer in a new free-energy minimum. In other words, the deletion may affect the intramolecular interactions so that the unfavorable loss of entropy accompanying the transition from monomer to dimer can be compensated by an enthalpic gain due to more effective intermolecular interactions.

Overall, these results show that in an evolutionary process of protein oligomerization, the "primary mutation", irreversibly leading to a monomer to dimer transition, can be the single deletion of a few residues in a critical region of the protein.

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