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Hints on the evolutionary design of a dimeric RNase with *special* bioactions*

ALBERTO DI DONATO, VALERIA CAFARO, IVANA ROMEO, AND GIUSEPPE D'ALESSIO

Dipartimento di Chimica Organica e Biologica, Università di Napoli Federico II. Via Mezzocannone, 16. 80134 Naples, Italy

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Abstract

Residues P19, L28, C31, and C32 have been implicated (Di Donato A, Cafaro V, D'Alessio G, 1994, *J Biol Chem 269*:17394–17396; Mazzarella L, Vitagliano L, Zagari A, 1995, *Proc Natl Acad Sci USA*: forthcoming) with key roles in determining the dimeric structure and the N-terminal domain swapping of seminal RNase. In an attempt to have a clearer understanding of the structural and functional significance of these residues in seminal RNase, a series of mutants of pancreatic RNase A was constructed in which one or more of the four residues were introduced into RNase A. The RNase mutants were examined for: (1) the ability to form dimers; (2) the capacity to exchange their N-terminal domains; (3) resistance to selective cleavage by subtilisin; and (4) antitumor activity. The experiments demonstrated that: (1) the presence of intersubunit disulfides is both necessary and sufficient for engendering a stably dimeric RNase; (2) all four residues play a role in determining the exchange of N-teminal domains; (3) the exchange is the molecular basis for the RNase antitumor action; and (4) this exchange is not a prerequisite in an evolutionary mechanism for the generation of dimeric RNases.

Keywords: antitumor; domain exchange; mutagenesis; protein engineering; ribonuclease

The fact that seminal ribonuclease (BS-RNase) has access to two quaternary conformations, in equilibrium with each other (Piccoli et al., 1992), is perhaps the most interesting of the unusual properties of this ribonuclease. BS-RNase is a dimer (see Kinemage 1), whereas the other RNases of the same superfamily are monomers, and two disulfides reinforce the noncovalent links between the two subunits (Di Donato & D'Alessio, 1973; D'Alessio et al., 1975). As an enzyme, BS-RNase displays a mixed type of cooperativity in the rate-limiting reaction step (Piccoli et al., 1988) and also efficiently degrades dsRNA (Libonati & Floridi, 1969). Furthermore, BS-RNase is endowed, like other RNases of the so-called RISBASE (*RI*bonucleases with *S*pecial *B*iological *A*ctions) group, with interesting bioactions (D'Alessio et al., 1991), including an antitumor action (Vescia et al., 1980; Laccetti et al., 1992, 1994). But most characteristic is the co-existence in the native enzyme of two dimeric forms (Piccoli et al., 1992): the form M × M, in which the two subunits exchange or "swap" (Bennett et al., 1994) their N-terminal tails (Mazzarella et al., 1993), and the form M = M, in which no swapping takes place, and each monomer folds onto itself (Fig. 1). The exchange of parts between BS-RNase subunits is not a mere structural feature, because the M × M dimeric form is the only form with the allosteric regulation of the rate-determining RNase reaction step (Piccoli et al., 1992) and is the more active form as an antitumor agent (Cafaro et al., 1995).

The apparent relationship between the co-existence in native BS-RNase of two dimeric forms and the interesting functional properties of the protein prompted an investigation of the structural determinants of this dual quaternary structure aimed at their identification and at the elucidation of their roles in eventually determining the antitumor action of the protein.

As a reference molecule for this investigation, we used RNase A, the prototype of the vertebrate RNase superfamily, a logical candidate because it (1) is monomeric, (2) is devoid of any of the special biological functions characteristic of BS-RNase, but (3) has more than 80% sequence identity with BS-RNase subunit chain (Suzuki et al., 1987). The choice was also based on the recent finding (Di Donato et al., 1994) that RNase A can be made dimeric, with a dual quaternary structure and also active as an antitumor agent by substituting into its polypeptide chain four of its residues with the residues present at the same positions in BS-RNase: Pro for Ala, Leu for Gln, and Cys for Lys and Ser, at positions 19, 28, 31, and 32, respectively (see Kinemage 1). These residues were chosen because of their in-

Reprint requests to: Giuseppe D'Alessio, Dipartimento di Chimica Organica e Biologica, Università di Napoli Federico II. Via Mezzocannone, 16. 80134 Naples, Italy.

*This paper is dedicated to the memory of William H. Stein and Stanford Moore.

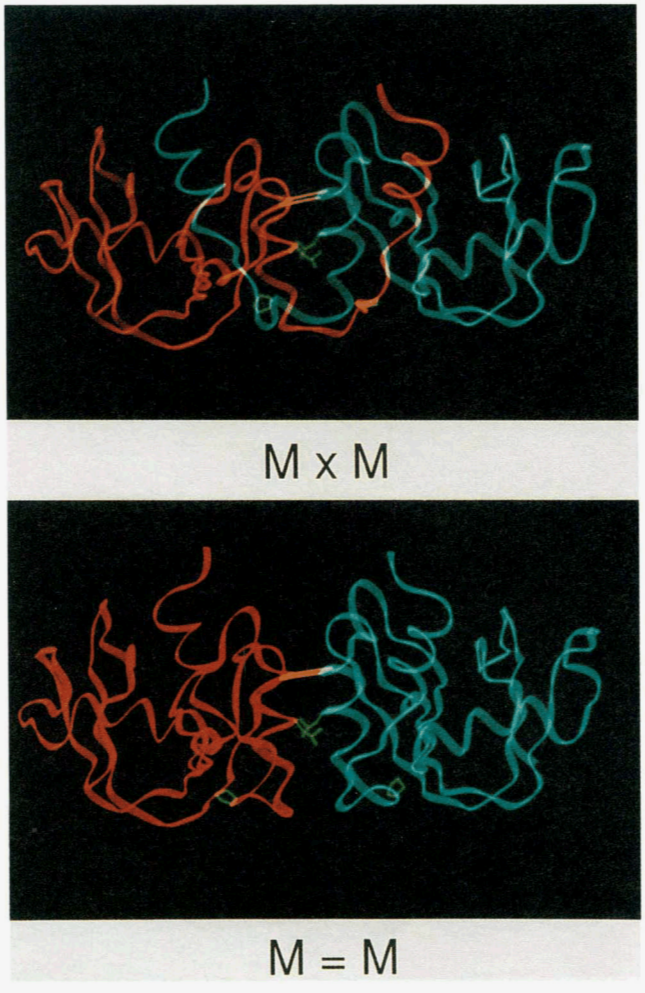

Fig. 1. Structure of BS-RNase, with exchange (form M × M) and with no exchange (form M = M) of the N-terminal domains. Side chains of residues P19, L28, C31, and C32 are highlighted.

volvement with key roles in determining the dimeric structure of BS-RNase (Mazzarella et al., 1993).

A series of mutants was constructed, to insert into the RNase A chain only one, two, or three of the four residues (P19, L28, C31, and C32) that have been shown (Di Donato et al., 1994) to be sufficient to provide RNase A with the structural and functional properties of interest. These single, double, and triple mutants of RNase A were tested for: (1) the ability to form dimers; (2) the capacity to exchange their N-terminal domains; (3) their resistance to selective cleavage by subtilisin; and (4) antitumor activity. Surprisingly, the experiments revealed that all four residues play a role in determining the domain-swapping property of the molecule. This was confirmed to be the molecular basis for the antitumor action of the protein. Moreover, on the basis of these results, it could be reaffirmed (D'Alessio, 1995) that, at least for RNases, domain swapping is not an evolutionary mechanism that drives dimerization, as suggested earlier (Bennett et al., 1994).

Table 1. *Nomenclature of the mutants of RNase A*

Mutant	Abbreviation
(A19P, Q28L, K31C, S32C) *dimeric* RNase A	PLCC-RNase AA
(A19P, K31C, S32C) *dimeric* RNase A	PCC-RNase AA
(Q28L, K31C, S32C) *dimeric* RNase A	LCC-RNase AA
(K31C, S32C) *dimeric* RNase A	CC-RNase AA
(A19P, Q28L, K31C, S32C) RNase A	PLCC-RNase A
(A19P, K31C, S32C) RNase A	PCC-RNase A
(Q28L, K31C, S32C) RNase A	LCC-RNase A
(K31C, S32C) RNase A	CC-RNase A
(A19P) RNase A	P-RNase A
(A19P, Q28L) RNase A	PL-RNase A

Results

Characterization of mutants

Table 1 summarizes the series of mutants of RNase A analyzed in this study. Following the guidelines for the construction of the tetramutant PLCC-RNase AA (Di Donato et al., 1994), mutations were inserted into the RNase A cDNA to introduce at one or more of the positions 19, 28, 31, and 32 of the protein chain the residues present at the same positions in BS-RNase (see Materials and methods). The production and purification of the dimeric mutants (the AA mutants) were possible due to the two-step procedure first described for the preparation of recombinant BS-RNase (de Nigris et al., 1993). As illustrated in Figure 2, recombinant RNase chains were first refolded in the

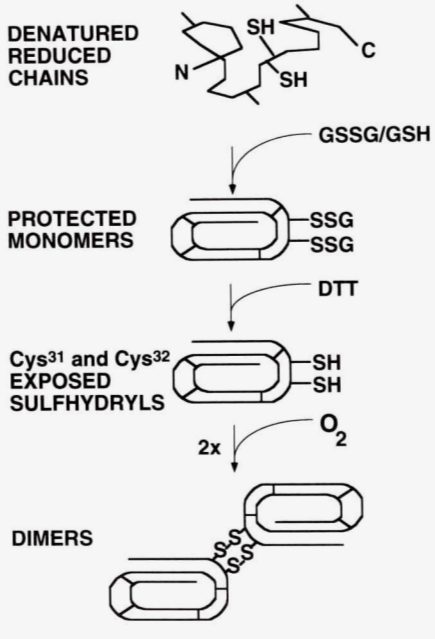

Fig. 2. Scheme for the preparation of recombinant dimeric mutants of RNase A. Denatured, fully reduced RNase chains are refolded in the presence of a GSH/GSSG redox mixture to yield monomeric intermediates in which the Cys residues at positions 31 and 32 are protected by glutathione moieties. Dimeric RNases AA are obtained by the removal of the glutathione through selective reduction with DTT, followed by air oxidation.

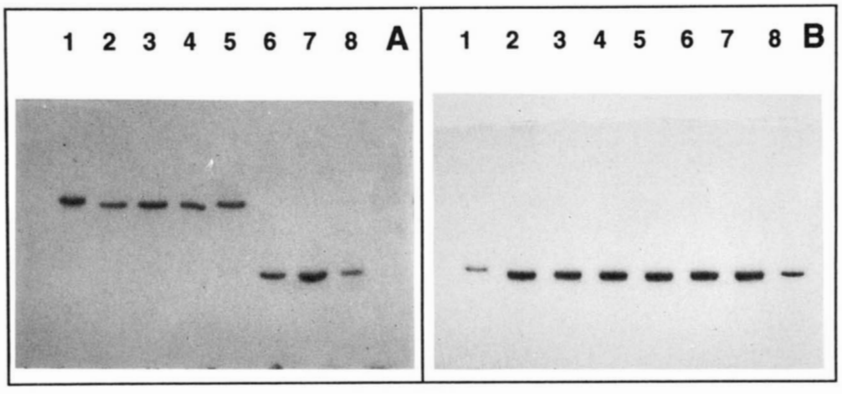

Fig. 3. SDS-PAGE electrophoresis in 15% polyacrylamide gels carried out under (**A**) nonreducing and (**B**) reducing conditions. Lanes 1–8, BS-RNase, PLCC-RNase AA, PCC-RNase AA, LCC-RNase AA, CC-RNase AA, P-RNase A, PL-RNase A, RNase A.

presence of a GSH/GSSG mixture to yield monomeric intermediates (mutants PLCC-, PCC-, LCC-, and CC-RNase A), in which the Cys residues responsible in BS-RNase for intersubunit disulfides were stably protected by glutathione moieties. Dimeric RNases AA were then obtained by the removal of the glutathione moieties through selective reduction of the mixed disulfides, followed by air oxidation of the exposed cysteines.

SDS-PAGE analyses revealed that all the recombinant proteins were homogeneous. As shown in Figure 3 the dimeric mutants PLCC-, LCC-, PCC, and CC-RNase AA have the molecular size of a dimeric RNase, such as BS-RNase, whereas the apparent molecular size of P- and PL-RNase A corresponds to that of monomeric RNase A. When denatured in the presence of 2-mercaptoethanol, the molecular size of the dimeric mutants PLCC-, LCC-, PCC-, and CC-RNase AA is halved, as occurs for BS-RNase after reduction of the intersubunit disulfides.

Residue substitutions in the mutant proteins were confirmed by sequence analyses conducted three to four residues downstream of the last mutated residue. The analyses indicated (data not shown) that, in all the mutants, the N-terminal residue was methionine, followed by the residues of RNase A, except for the mutated amino acid(s).

The catalytic activity of the mutants was tested on RNA as a substrate (Table 2). All the dimeric mutants displayed reduced specific activity with respect to native, monomeric RNase A. This is reminiscent of the case of dimeric BS-RNase, which under the same conditions has a specific activity about half of that of its monomeric form (Piccoli & D'Alessio, 1984). The mono-

Table 2. *Properties of the mutants of RNase A*

Protein	Specific activity[a]	$\Delta G°$ (kcal/mol)[b]	Crestfield-type dimers (%)[c]	Antitumor activity (%)[d]	Sensitivity to subtilisin[e]
RNase A	100		17	0	100
PLCC-RNase AA	63.5	−0.35		40.6	n.d.[f]
PCC-RNase AA	65.5	+0.44		29.0	n.d.
LCC-RNase AA	61.3	+0.78		19.5	n.d.
CC-RNase AA	69.4	+1.17		12.2	n.d.
PLCC-RNase A	119.3		14.9		6.9
PCC-RNase A	96.3		19.2		5.2
LCC-RNase A	95.3		9.5		31.7
CC-RNase A	96.2		7.8		23.9
PL-RNase A	127.1		14.7		40.1
P-RNase A	116.3		17.7		33.4
BS-RNase[g]	45	−0.41		80	0

[a] Relative to RNase A specific activity, taken as 100. The activity of RNase A ranged between 95 and 108 Kunitz units/mg of protein (Kunitz, 1946).

[b] $\Delta G°$ values for the formation of M × M species were calculated from the equilibrium concentrations of the M × M and M = M forms.

[c] Percentage of the RNase recovered in the dimeric fraction after lyophilization from acetic acid solutions (Crestfield at al., 1962).

[d] Cell proliferation after 72 h of growth (see Materials and methods), normalized to 100%, is expressed as 0% of antitumor action; an antitumor action of 100% would correspond to total cell death.

[e] Measured from the amount of S-peptide released from the protein under the assay conditions (see Materials and methods) and expressed as percentage of the amount of S-peptide released under identical conditions from RNase A, taken as 100%.

[f] n.d., Not determined.

[g] Data from Piccoli et al. (1992).

meric mutants instead were found to be as active, or even more active, than parent RNase A.

Quaternary conformation of dimeric mutants of RNase A

To assess whether the PCC-, LCC, and CC-RNase AA mutants equilibrated like BS-RNase (Piccoli et al., 1992) and PLCC-RNase AA (Di Donato et al., 1994) into the exchanging M × M and the nonexchanging M = M dimeric forms, a sample of each mutated protein was subjected to selective cleavage of the intersubunit disulfides (D'Alessio et al., 1975), followed by analysis of the products by gel filtration. Using this procedure, already described for BS-RNase (Piccoli et al., 1992) and PLCC-RNase AA (Di Donato et al., 1994), nonexchanging dimers (M = M type) readily dissociate into monomers upon reduction, and elute as monomers from a gel filtration column, whereas exchanging dimers (M × M type) do not dissociate and elute as noncovalent dimers.

Figure 4 shows that all the RNase AA mutants, as isolated, had different contents of the M × M-type dimers, which increased upon incubation at pH 7 at 37 °C, i.e., under conditions that induce the transformation of the BS-RNase M = M form into M × M (Piccoli et al., 1992). After about 200 h, for all mutants, the M × M form reached an equilibrium with the M = M form. As shown in Figure 4, the content of the M × M form at equilibrium is lower in the triple mutants LCC- and PCC-RNase AA, and even lower in the CC-RNase AA mutant, with respect to that of the tetramutant PLCC-RNase AA, the only mutant for which a full content of about 65% of the M × M form, i.e., that found for native BS-RNase (Piccoli et al., 1992), was found.

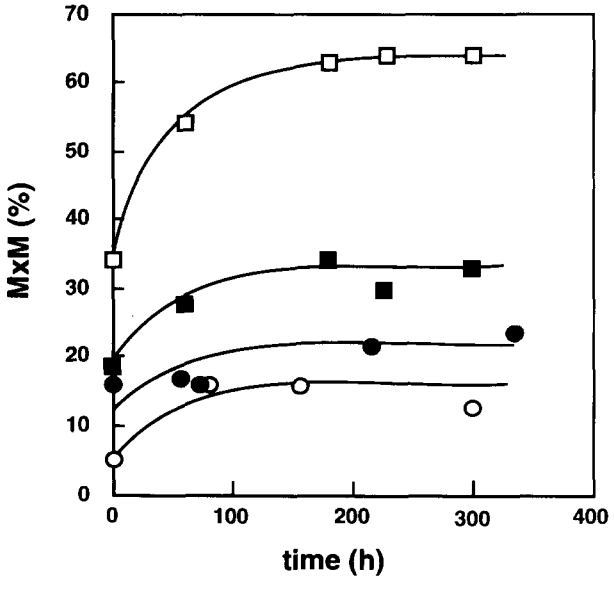

Fig. 4. Equilibration of RNase AA mutants (□, PLCC-; ■, PCC-; ●, LCC; ○, CC-RNase AA) into the M × M and M = M quaternary conformations. Proteins were incubated at 37 °C in 0.1 M Tris-acetate, pH 7.0, at a concentration of 0.8 mg/mL. At the indicated time intervals, aliquots were withdrawn and analyzed by gel filtration after cleavage of the intersubunit disulfides (see Materials and methods). Percentage of the M × M form present at each time interval was plotted.

Effect of lyophilization from acetic acid solutions

The classic pioneering experiments by Crestfield and colleagues (Crestfield et al., 1962) showed that monomeric bovine pancreatic RNase A associates, upon freeze-drying from acetic acid solutions, into metastable dimers of the M × M type. We thus used the same experimental tool to address the question of whether or not the mutated RNase A monomers also possessed an intrinsic propensity to dimerize, and if this was different from that of their parental protein.

PLCC- and PCC-RNase A monomers and monomeric P- and PL-RNase A mutants, subjected to lyophilization from acetic acid solutions, were found to be capable of association into Crestfield-type dimers (see Table 2) to an extent similar to that of wild-type RNase A, whereas mutant CC- and LCC-RNase A, both lacking a Pro residue at position 19, associated under the same conditions in a much lower proportion.

Effect of subtilisin

When RNase A is treated with subtilisin under controlled conditions, a selective proteolytic cleavage occurs at the N-terminal domain of the protein, mainly between residues 20 and 21 (Richards & Vithayathil, 1959). This is the domain that is swapped between subunits in the M × M dimers, as present in BS-RNase, or produced by lyophilization of RNase A from acetic acid, or between monomers of RNase AA mutants. RNase A dimers, obtained by freeze-drying from acetic acid solutions, are resistant to the action of subtilisin (Libonati et al., 1973) as are naturally dimeric BS-RNase and artificial monomers of BS-RNase (Parente et al., 1976). It has been proposed (Parente et al., 1976) that the substitution in BS-RNase at position 19 of Pro for Ala, present at that position in RNase A, makes the conformation of the N-terminal domain of the molecule incompatible with the active site of subtilisin.

Thus, subtilisin was used as a structural probe for the conformation of the N-terminal domain of the mutant RNases tested under the conditions in which RNase A is selectively and effectively cleaved (see Materials and methods). The amount of protein cleaved by the action of subtilisin was measured by the amount of S-peptide (1-20) released by the protease. The results of the experiments, shown in Table 2, indicate that all the mutants have a much lower sensitivity to subtilisin selective cleavage than RNase A. Especially resistant were the monomeric mutants PCC- and PLCC-RNase A, which share the two Cys residues at positions 31 and 32, and a Pro residue at position 19.

Antitumor activity of the RNase A mutants

The antitumor action of mutated ribonucleases was tested by measuring the percentage of survival of SVT2 fibroblasts after 72 h of growth in the presence of 50 μg/mL of the proteins being tested.

Given the dependence of the antitumor activity of BS-RNase on its quaternary conformation (Cafaro et al., 1995), i.e., on the presence of the more active M × M forms, the measurement of the cytotoxicity of the dimeric mutants was carried out using protein preparations that were incubated for 300 h under conditions that induce the transformation of the M = M form into, and its equilibration with, M × M (see above and Fig. 4). All protein preparations were thus tested for their M × M content

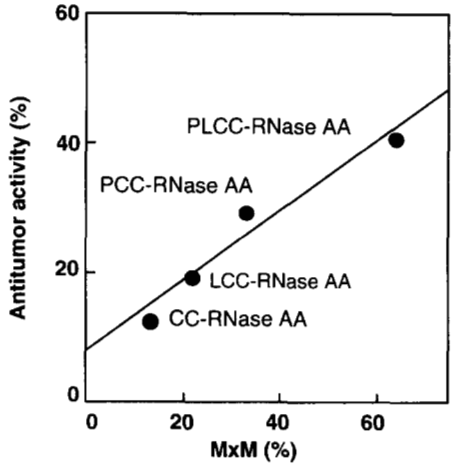

Fig. 5. Relationship between antitumor activity and the percentage of the M × M form of dimeric RNases AA. Data fit a linear relationship with a correlation coefficient of 0.98.

before they were subjected to the antitumor assay. The results of the assays for antitumor activity, correlated with the M × M content of all the dimeric mutants of RNase A, are shown in Figure 5. They confirm the direct correlation between the cytotoxicity of an RNase molecule and its quaternary conformation.

Discussion

The dimeric structure of BS-RNase is unique in the whole vertebrate RNase tree. This in particular indicates the co-presence in a single genome of two homologous, very similar genes, coding for proteins with the same enzymic function but with distinct structural fates: one a dimer, the other a monomer. The RNase peptide chain expressed in bovine pancreas folds as a monomeric protein (Blackburn & Moore, 1982), whereas the highly similar RNase chains (with about 18 significant substitutions over 124 residues) expressed in the seminal vesicles of the same bovine species fold and associate into dimers (D'Alessio et al., 1991). Because four of these substitutions are sufficient to transform RNase A into a dimeric RNase (Di Donato et al., 1994; Mazzarella et al., 1995), we scrutinized these four residues and investigated their roles in the RNase dimerization process. We tried to answer the question: which are the minimal determinants for the spontaneous, stable dimerization of a monomeric RNase?

Dimeric BS-RNase also has the unusual property of equilibrating in two different quaternary conformations, M = M and M × M (Piccoli et al., 1992). Because the above-mentioned dimeric mutant of RNase A acquires, with the ability to dimerize, the same property (Di Donato et al., 1994), we also investigated the roles of those four residues in determining the dual quaternary conformation of the RNase. The question was: which are the determinants for conferring to a dimeric RNase the structural motif consisting in the exchange of the N-terminal α-helices between subunits?

The answer that our data provided to the first question was straightforward and not surprising: the double CC-RNase A monomeric mutant, upon removal of the (quite physiological) glutathione blocking groups, promptly and completely dimerized into CC-RNase AA. Thus, an RNase chain predetermined to fold into a monomer can be transformed into a stable, catalytically active, dimeric RNase when two Cys residues are present at positions 31 and 32 of its sequence, as in naturally dimeric BS-RNase (Suzuki et al., 1987) (see Kinemage 1). At present, it cannot be excluded that the insertion in RNase A of a single Cys at either position is sufficient to determine the same effects. However, recent data (Kim & Raines, 1994) point out that anomalous dimers are produced when a single intersubunit disulfide links BS-RNase subunits, and refolding is obtained by air reoxidation of the intersubunit disulfides.

The answer to the second question, on the swapping determinants, was more intriguing. The analysis was based on the data on the swapping ability of each dimeric mutant and on two other lines of evidence, albeit indirect. These resulted from the use of subtilisin as a probe of the conformation of the N-terminal domain of the monomeric mutants, and from testing the antitumor property of the dimeric RNases, clearly based on a specific molecular recognition between the RNase and some cellular structure(s). RNase A and BS-RNase were precious reference points for the interpretation of the data: the former with no antitumor activity and susceptible to selective cleavage by subtilisin; the latter with antitumor activity, correlated with its swapped M × M conformation (Cafaro et al., 1995) and totally resistant to subtilisin, even in its monomeric form (Parente et al., 1976).

When the data obtained with the mutants lacking P19 (CC and LCC mutants) were compared with those obtained with the corresponding PCC and PLCC mutants (see Figs. 4, 5), the role of P19 (located in BS-RNase in the hinge region that links the "swappable" N-terminal domain with the main subunit domain) became apparent (see Kinemage 1). In PCC- compared with CC-RNase AA, and in PLCC-compared with LCC-RNase AA: (1) the extent of swapping increases from 13 to 33% and from 22 to 64%, respectively, and (2) the antitumor activity increases from 12 to 29% and from 20 to 41%, respectively. In the corresponding monomeric mutants (PCC- compared to CC-RNase A, and PLCC- compared to LCC-RNase A), the sensitivity to subtilisin decreases from 24 to 5% and from 30 to 7%, respectively. Also, the other monomeric mutants endowed with the P19 mutation, P- and PL-RNase A, have a dramatically decreased sensitivity to subtilisin cleavage with respect to wild-type RNase A.

Thus, the insertion of a Pro residue at position 19 alters dramatically the conformation of the hinge region and enhances the propensity to swapping the N-terminal domains. These conclusions are in line with the conclusion of Mazzarella et al. (1995), in that P19 is to be assigned in BS-RNase the role of a "swapping determinant."

However, even in the CC-RNase AA mutant, a low but significant extent of exchange (about 13%) of the N-terminal domains could be measured; furthermore, the Q28L mutation was found to have by itself a significant effect. This is evident from the comparison of the stability of the M × M form of CC-RNase AA ($\Delta G^\circ = +1.17$ kcal/mol) with that of the M × M form of LCC-RNase AA ($\Delta G^\circ = +0.78$ kcal/mol), and from the stability of the M × M form of PCC-RNase AA ($\Delta G^\circ = +0.44$ kcal/mol) compared to that of the M × M form of PLCC-RNase AA ($\Delta G^\circ = -0.35$ kcal/mol). Thus, the presence of a Leu at position 28 and/or of two Cys residues at positions 31 and 32, i.e., at positions quite distant from the hinge region, increases the stability of the M × M form of a dimeric RNase (see Kine-

mage 1). These data can be interpreted by recognizing that the stronger the contacts at the subunit interface, the more the swapping is facilitated. We propose that the interface between the monomers of a dimeric RNase can be visualized as a fulcrum for the displacement and for the reciprocal movements of the two N-terminal domains. Two disulfide bonds make a stable interface, so that, in CC-RNase AA, the M × M conformation can in turn be stabilized; the addition with Leu-28 of a hydrophobic contact (Mazzarella et al., 1993) makes it even more stable. On the other hand, addition of a Pro residue at position 19 (as in PLCC-RNase AA) also contributes, because it renders the hinge peptide energetically more stable (Mazzarella et al., 1995) in the M × M conformation.

Hence, a comprehensive conclusion is that all four residues have a role in determining the stability of the M × M conformation; they all are "swapping determinants."

Surprisingly, although the role of P19 as a "swapping determinant" appears to be well established, its presence in the RNase A chain does not notably increase the propensity of RNase monomers to swap their N-terminal ends through lyophilization in acetic acid. This indicates that the ability of RNase monomers to swap their N-terminal ends and yield the metastable Crestfield-type dimers cannot be related to the swapping ability of the corresponding RNase dimers stabilized by covalent crosslinks. As a consequence, the former property cannot be considered an experimental basis to support the hypothesis that domain swapping between subunits is a mechanism in the evolutionary path from monomeric to oligomeric RNases (Bennett et al., 1994). In fact, the data on the latter type of domain swapping, i.e., between subunits of stable dimers, also do not support this hypothesis as anticipated (D'Alessio, 1995). All the CC mutants (CC-, PCC-, LCC-, PLCC-RNase AA) capable of making intersubunit disulfides are stable dimers, but they show quite different abilities in swapping their N-terminal domains. Hence, all the data reported here appear to lead to the conclusion that, at least for the making of an oligomeric vertebrate RNase, the accumulation of mutations at a certain surface of a monomeric protein, the eventual subunit interface, can still be regarded as the most feasible way for the evolution from monomeric to oligomeric proteins.

The experimental data reported above also confirm recent data (Cafaro et al., 1995) on the importance of domain swapping for the antitumor action of BS-RNase. A correlation between extent of domain swapping and extent of antitumor action in the RNase A mutants is apparent from the data summarized in Table 2. It seems to be strengthened by the presentation illustrated in Figure 5: from the extrapolation of the linear correlation ($r = 0.98$) between the two sets of data, it could be speculated that a dimer in which no N-terminal swap occurred would be virtually devoid of antitumor action.

Materials and methods

General procedures

BS-RNase was purified from seminal vesicles as described by Tamburrini et al. (1986).

Bacterial cultures, plasmid purifications, and transformations were performed according to Sambrook et al. (1989). Double-strand DNA was sequenced with the dideoxy method of Sanger et al. (1977), carried out with a Sequenase sequencing kit (US Biochemical Corporation) with deoxynucleotide triphosphates purchased from Pharmacia. The cDNA coding for RNase A was prepared as described (Di Donato et al., 1993) starting from a *Bam*H I/*Eco*R I 1,141-bp fragment containing the entire coding sequence of pancreatic RNase A, provided by Dr. Adriana Furia (University of Naples). The expression vector pT7-7 was provided by Dr. Gennaro Ciliberto (University of Naples). Plasmid pUC18 and *Escherichia coli* strain JM101 were purchased from Boehringer; *E. coli* strain BL21(DE3)pLysS from AMS Biotechnology; labeled oligonucleotides from Amersham. The Gene-Clean kit for elution of DNA fragments from agarose gel was obtained from Bio 101. Enzymes, including restriction endonucleases, and other reagents for DNA manipulation, were from Promega Biotech.

Site-directed mutagenesis

The desired mutations were introduced in the RNase A cDNA cloned into plasmid pUC118 between *Eco*R I and *Sal* I sites as described (Di Donato et al., 1994). The resulting plasmid was mutated according to Kunkel (1985) using the appropriate mutagenic oligonucleotides synthesized by Beckman Analytical (Italy). The mutations were always verified by DNA sequencing.

Protein expression and purification

The cloned cDNAs, excised from the mutant plasmids with the appropriate restriction enzymes, were isolated by agarose gel electrophoresis, inserted into the pT7-7 expression vector, and expressed as described previously (de Nigris et al., 1993; Di Donato et al., 1994). As described in Figure 2, the mutant proteins with Cys residues at position 31 and 32 were dissolved from inclusion bodies and unfolded in 6 M guanidine-Cl, purified by gel filtration, and refolded in the presence of a glutathione redox buffer. This yielded catalytically active monomeric RNase mutants with mixed disulfides protecting the cysteine residues at positions 31 and 32 of the chain. Dimerization of these RNase monomers was obtained after the removal of the glutathione moieties by selective reduction with DTT (de Nigris et al., 1993), followed by dialysis against 0.1 M Tris-Cl, pH 8.4, and gel filtration on a HiLoad Superdex 75 column. Yields of dimers ranged from 5 to 10 mg/L bacterial culture.

Determination of M × M and M = M content of protein preparations

The contents of M × M and M = M forms in dimeric mutant RNases were determined as previously described (Piccoli et al., 1992). Briefly, about 10 μg of each protein, dissolved in 20 μL of 0.1 M Tris-Cl, pH 8.4, was reduced under a nitrogen barrier in the presence of a 15-fold molar excess of DTT for 20 min at room temperature. This ensures the selective reduction of intersubunit disulfides (D'Alessio et al., 1975). Samples were then diluted to 100 μL with 50 mM ammonium acetate, pH 5.0, containing 0.3 M NaCl, and directly injected into a gel filtration HiLoad 10/30 Superdex 75 column, eluted with the same buffer, at a flow rate of 0.3 mL/min. The absorbance of the eluate was recorded at 214 nm, using a Shimadzu model CR6-A recorder/integrator. The area of the peaks was read directly from the printout of the integrator.

Lyophilization from acetic acid solutions

Each mutant was dissolved at a concentration of 0.6 mg/mL and lyophilized from a 50% acetic acid solution containing 10 mM sodium phosphate, pH 7.2, as described (Crestfield at al., 1962). The lyophilized material was gel-filtered on a Sephadex G-75 Superfine column (0.9 × 47.5 cm) equilibrated with 10 mM sodium phosphate, pH 7.2, at a flow rate of 2 mL/h. The absorbance of the eluate was monitored at 230 nm and the percentage of dimeric material was obtained by integrating the area of the peak eluting at the elution volume corresponding to that of dimeric BS-RNase.

Subtilisin cleavage

Incubations were performed in 0.1 M Tris-Cl, pH 8.0, at a protein concentration of 1 mg/mL, with a weight ratio of subtilisin to protein substrate of 1:100 (Richards & Vithayathil, 1959), in a volume of 200 μL. After 2 h at 0 °C, the incubates were acidified with HCl and applied to a reverse-phase Vysac C_4, 5-μm column (0.45 × 25 cm), equilibrated in 95% solvent A (0.1% trifluoroacetic acid in H_2O) and 5% solvent B (0.1% trifluoroacetic acid in acetonitrile). The elution was carried out at a flow rate of 1 mL/min; 10 min after loading the samples, the concentration of solvent B was increased to 40% over 60 min. The absorbance of the eluate was recorded at 214 nm, using a Shimadzu model CR6-A recorder/integrator. The extent of cleavage was determined by integrating the area of the peak eluting at the elution volume of the S-peptide released by the action of subtilisin from a sample of homogeneous RNase A.

Antitumor assays

The antitumor action of the mutant RNases was measured from their selective cytotoxic effect on tumor cells. In the absence of added RNase, cell proliferation, normalized to 100%, was expressed as 0% of antitumor action, whereas an antitumor activity of 100% corresponded to total cell death. Selective cytotoxicity was ascertained by using as normal cells BalbC 3T3 fibroblasts, obtained from ATCC (Richmond, USA), and as malignant cells the same 3T3 line transformed with SV40 virus (Vescia et al., 1980). The cells were cultured in 24-well plates at a density of 50 × 10^3 per mL of Dulbecco's modified Eagle medium, supplemented with glutamine, penicillin, streptomycin, and 10% fetal calf serum. The RNases being tested were added to the cells before plating at a concentration of 50 μg/mL. Surviving cells were counted after 72 h of growth, which allowed at least 3–4 rounds of replication in the control cultures. All the RNases produced and tested in this investigation were found to have no effect on normal 3T3 cells.

Other methods

RNase activity on yeast RNA was assayed with the method of Kunitz (1946). SDS-PAGE was carried out according to Laemmli (1970). Protein sequence determinations were performed on an Applied Biosystems sequencer model 473A, connected on-line with the HPLC apparatus for identification of phenylthiohydantoin derivatives.

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