REVIEW ARTICLE

Oligomerization of bovine ribonuclease A: structural and functional features of its multimers

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Bovine pancreatic RNase A (ribonuclease A) aggregates to form various types of catalytically active oligomers during lyophilization from aqueous acetic acid solutions. Each oligomeric species is present in at least two conformational isomers. The structures of two dimers and one of the two trimers have been solved, while plausible models have been proposed for the structures of a second trimer and two tetrameric conformers. In this review, these structures, as well as the general conditions for RNase A oligomerization, based on the well known 3D (three-dimensional) domain-swapping mechanism, are described and discussed. Attention is also focused on some functional properties of the RNase A oligomers. Their enzymic activities, particularly their ability to degrade double-stranded RNAs and polyadenylate, are summarized and discussed. The same is true for the remarkable antitumour activity of the oligomers, displayed *in vitro* and *in vivo*, in contrast with monomeric RNase A, which lacks these activities. The RNase A multimers also show an aspermatogenic action, but lack any detectable embryotoxicity. The fact that both activity against double-stranded RNA and the antitumour action increase with the size of the oligomer suggests that these activities may share a common structural requirement, such as a high number or density of positive charges present on the RNase A oligomers.

Key words: antitumour activity, double-stranded RNA, ribonuclease A, RNase A oligomer, single-stranded RNA, three-dimensional domain swapping.

INTRODUCTION

Protein aggregation, the ways in which this event may occur and the variables influencing it are at present interesting and important topics of research, not only from a purely scientific point of view, but also for practical reasons. Many neurodegenerative diseases of humans and animals are characterized by the finding of aggregated forms of proteins localized inside the cytoplasm or the cell nucleus, or even between neurons. β-Amyloid is found in Alzheimer's disease, and α-synuclein deposits to form Lewy bodies are commonly associated with Parkinson's disease. A modified form of the prion protein is responsible for transmissible human and animal encephalopathies, including Creutzfeldt–Jakob disease, bovine spongiform encephalopathy and scrapie in sheep [1]. Moreover, the so-called polyglutamine expansion diseases (Huntington's disease, Kennedy's disease etc.) are also characterized by the presence of insoluble, granular and fibrous protein deposits in the nuclei of the affected neurons [2]. On the other hand, experimental results suggest that every protein can aggregate or even form amyloid fibrils provided that its concentration is high enough and appropriate environmental conditions are chosen to destabilize its native structure [3–5]. Much work has also been carried out in an attempt to identify the characteristics of a protein that are responsible for its propensity to form amyloid structures, and the factors influencing this process [6–9], among which the solubility of a polypeptide appears to be very important [10].

Models for the experimental investigation and increased understanding of the mechanisms leading to protein aggregation can be quite useful. In this regard, bovine pancreatic RNase A (ribonuclease A) is an appropriate model because of its remarkable structural and functional versatility. Although it has been, over the years, one of the most studied proteins, this rather small and very stable enzyme continues to reveal new, interesting and sometimes surprising properties. The classic experiment described more than 40 years ago by Crestfield, Stein and Moore [11] was the starting point of studies on RNase A aggregation: by lyophilization from 50 % (v/v) acetic acid solutions, RNase A was found to form two dimeric conformers that were characterized and shown to be enzymically active [11–13]. In connection with this, it might be worth pointing out here a notable difference between the meaning of protein aggregation in the case of pathological events and the aggregation of RNase A, which we will talk about here. The term 'aggregation' referred to a protein may suggest insolubility, loss of function and therefore abnormality, a concept that applies particularly to the protein aggregates found in the nervous tissue of people afflicted by neurodegenerative diseases. On the other hand, all aggregates of bovine RNase A are highly soluble and biologically active molecules, like all aggregates of the approximately 40 proteins that oligomerize through a 3D (three-dimensional) domain-swapping mechanism [14]. Therefore, to avoid misunderstandings, from now on we will generally prefer the term 'oligomerization' to indicate the 'aggregation' of RNase A.

Many years after the work of Crestfield, Stein and Moore [11], several different types of oligomers of RNase A in addition to the two dimers, ranging from trimers to pentamers and possibly larger oligomers [15], with each species existing as two (or more) conformational isomers [16], were shown to form not only by lyophilization from aqueous acetic acid solutions [11–13,15,16], but also under different experimental conditions without lyophilization [17]. The two dimers and one of the two trimers have been crystallized and their structures solved [3,18,19], and plausible models have been proposed for the second trimer

Abbreviations used: BS-RNase, bovine seminal RNase; cRI, cytosolic ribonuclease inhibitor; 3D, three-dimensional; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA.

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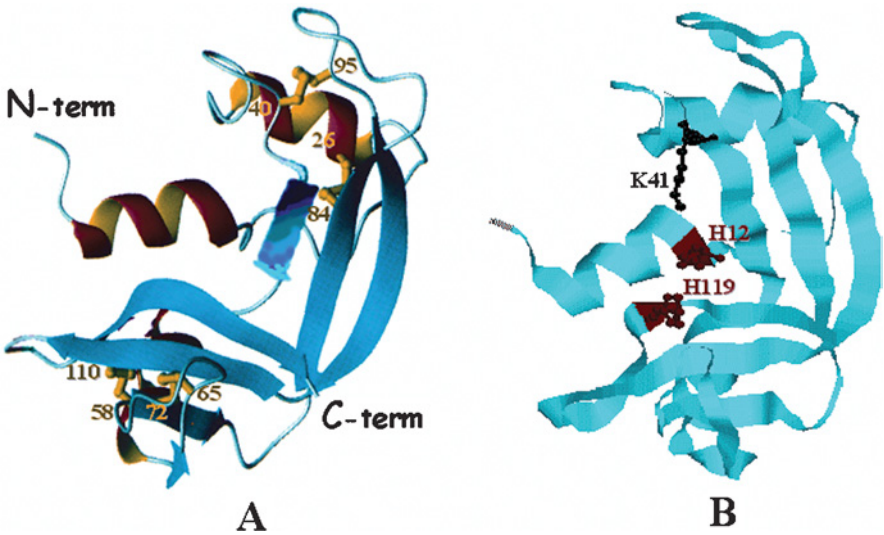

Figure 1 Bovine RNase A

PDB code 5RSA. (**A**) The four disulphide bridges (26–84, 40–95, 58–110 and 65–72) of the enzyme are shown as yellow sticks. (**B**) The catalytic triad of RNase A, shown in red and black balls and sticks.

and two tetramers [3,14,19,20]. The very low amounts of the larger aggregates (pentamers, hexamers?) formed has delayed, at least for the moment, the investigation of their structures in detail. Studies of the catalytic properties revealed that, upon oligomerization, RNase A acquires a depolymerase action on dsRNA (double-stranded RNA). More recent studies revealing a remarkable antitumour action of RNase A oligomers have rekindled the biological interest in the quaternary structures of these molecules. In this review we will briefly describe the structures of the main oligomers of RNase A, and their principal functional and biological properties.

BOVINE RNase A AND ITS OLIGOMERS: STRUCTURAL ASPECTS

RNase A

Before describing the products of the artificial oligomerization of bovine pancreatic RNase A (EC 3.1.27.5), a short description of some of the main features of RNase A is appropriate. The enzyme (Figure 1), which is one of the most thoroughly studied proteins in the world [21–24], is composed of 124 amino acids (molecular mass 13 686 Da [23]), among which basic residues (10 Lys, four Arg and four His) prevail over acidic residues (five Glu and five Asp), making the protein definitely basic, with a pI of 9.3. The protein's secondary structure consists of three α-helices and seven β-strands, and the molecule contains four disulphide bonds (26–84, 40–95, 58–110 and 65–72) that contribute, in particular the two terminal ones (26–84 and 58–110), to the remarkable stability of the protein [23,25]. It might be worth pointing out that RNase A, in contrast with many other proteins with disulphide bridges, has rather long termini outside the disulphide-bonded core, in which the two active-site histidines (His-12 and His-119) are indeed located. The N-terminus (residues 1–15), comprising one of the α-helices (residues 3–13), is connected to the body of the protein by a flexible loop (residues 16–23) that, under controlled conditions, may be cleaved by subtilisin, forming RNase S, without any loss of activity [21,26,27]. The C-terminal β-hairpin of RNase A is composed by two β-strands (residues 105–124) that are relatively richer in hydrophobic residues than the N-terminus. Both termini can become mobile under defined experimental conditions that weaken the non-covalent bonds between them and the protein core. The protein can be viewed as a 'kidney bean' whose two main lobes are separated by a positively charged region that binds the RNA substrate [24]. We concentrate here on some details of its enzymic activity that are relevant to the subsequent discussion, referring the interested reader to the many excellent reviews available for more detailed information concerning the structural and functional properties of RNase A [21–24].

The active site of the enzyme is formed by His-12, His-119 and Lys-41. The well known 'in-line' mechanism of the endonucleolytic cleavage of ssRNA (single-stranded RNA) by RNase A consists of an initial transesterification step (leading to 2′,3′-cyclic phosphate intermediates), and a subsequent hydrolytic action on these intermediates (to produce 3′-phosphomononucleotides and 3′-phospho-oligonucleotides ending in cytidine 3′-phosphate or uridine 3′-phosphate). To take place, the reaction requires that the 2′ oxygen of the ribose hydroxy group, which acts as an internal nucleophile, and the 5′ oxygen leaving group simultaneously occupy the two apical positions within the trigonal bipyramidal phosphorane intermediate [28]. This steric requirement makes a fruitful interaction of the enzyme with dsRNA impossible, and therefore it is chiefly responsible for the high resistance of this RNA species to attack by native, monomeric RNase A. It must be remembered, however, that other variables, such as ionic strength and the presence of carbohydrate chains on the enzyme protein, influence the resistance of double-helical RNA to ribonuclease attack [29,30].

Oligomerization of RNase A

All RNase A multimers presently known are formed by a 3D domain-swapping mechanism. This term was first introduced by David Eisenberg and his co-workers to describe the structure of the dimer of diphtheria toxin [31]. To date around 40 protein oligomers have been identified that are formed by the same mechanism [14], which consists of the exchange of identical domains (i.e. identical structural elements, usually the N- or C-termini of the protein, but sometimes also its middle domain)

between two or more protein monomers [14]. The model is similar to that advanced by Crestfield et al. [11], that was suggested to them by the results of a study by Vithayathil and Richards [32] on the inactivation of RNase S by iodoacetate. Crestfield, Stein and Moore [11] envisaged the model to explain the formation of dimeric RNase A during lyophilization from 50 % (v/v) acetic acid solutions of the protein partially unfolded by the acidic medium. It might be worth pointing out here that, while Fruchter and Crestfield [12] identified the two different dimeric conformers, they attributed the same mechanism of formation, i.e. the exchange of the N-terminus of one monomer with the N-terminus of the second monomer, to both of them. The difference between the two dimers was ascribed by Fruchter and Crestfield [12] to the different phosphate-binding capacities of the two conformers. The terminology and all structural details of 3D domain swapping can be found in a recent, excellent review by Liu and Eisenberg [14]. While the intimate mechanism of 3D domain swapping is still unclear, it is now well established that the domain-swapping event is independent of sequences and secondary structures, and that the hinge loops or linker regions are very flexible and arise from diverse sequences and secondary structures. 3D domain swapping is not limited to any particular structural or functional class of proteins: cases of proteins whose secondary structure is all α, α/β, $\alpha+\beta$ or all β have been reported; as well as various protein types such as toxins, enzymes, structural proteins and receptors [14].

All 3D-domain-swapped multimers identified so far, with one exception, are formed through the exchange of only the N-terminus or the C-terminus of a protein, and can be linear or circular structures [14]. The only interesting exception, for the present, is represented by the oligomers of RNase A, which can form by the swapping of the N-terminus, the C-terminus or both termini of the protein at the same time, with the production of linear and cyclic oligomers [3,14,18–20]. These observations indicate once again the extraordinary structural versatility of bovine pancreatic RNase A, whose possibilities of oligomerization surpass those of all other 3D-domain-swapping proteins presently known. Moreover, the possibility that a single protein can swap both of its termini indicates that the domain-swapping mechanism is more general than previously thought, and suggests a model for how higher-order polymers can form (see below).

The classic procedure described by Crestfield, Stein and Moore [11] to aggregate RNase A consisted of lyophilizing the protein from 50 % (v/v) acetic acid solutions. The lyophilized material was then dissolved in sodium phosphate buffer (pH 6.7) and applied on to a gel-filtration or cation-exchange column. Although their gel-filtration chromatograms revealed the presence of unresolved aggregated material with a molecular mass higher than 29000 Da (which roughly corresponds to dimeric RNase A [11]), Crestfield et al. only studied the two dimeric conformers of RNase A [11–13].

The investigation of the artificial oligomerization of RNase A was resumed many years later [15,16]. The patterns shown in Figures 2(A) and 2(B) show all of the aggregated species that can be identified by gel-filtration or, more efficiently, by ion-exchange chromatography of RNase A lyophilized from 40 % (v/v) acetic acid solutions [16]. They are dimers, trimers, tetramers, pentamers and possibly higher oligomers, with each species consisting of two (or more?) conformational isomers, one less basic and one more basic, all of which are enzymically active [16]. As the catalytic residues His-12 and His-119 are contributed by the N-terminal α-helix and the C-terminal β-strand respectively, oligomerization in fact leads to the formation of composite active sites.

The RNase A oligomers obtained by lyophilization from aqueous acetic acid solutions are produced in defined and rather

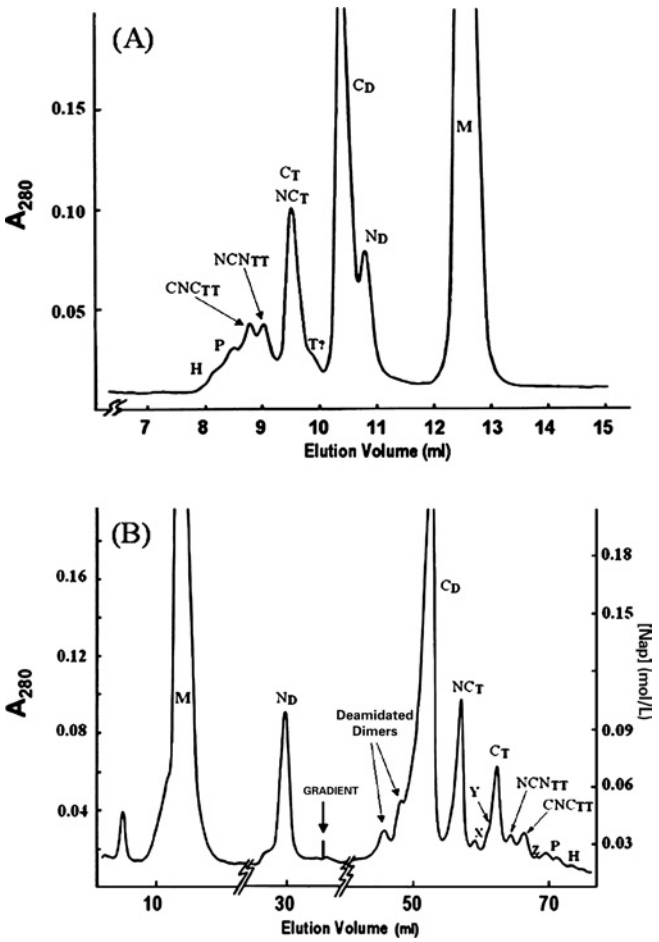

Figure 2 RNase A oligomers

(**A**) Gel-filtration chromatogram of RNase A lyophilized from a 40 % (v/v) acetic acid solution. H, putative hexamer; P, putative pentamer; CNC_{TT}, tetramer containing two swapped C-termini; NCN_{TT}, tetramer containing two swapped N-termini; C_T, NC_T, unresolved cyclic trimer (C-terminal swapped), plus linear trimer (containing both N-terminal and C-terminal swapping); T?, hypothetical additional trimeric oligomer; C_D, C-dimer (C-terminal swapped); N_D, N-dimer (N-terminal swapped); M, RNase A monomer. Experimental conditions were as described in [16]. (**B**) Cation-exchange pattern of RNase A lyophilized from a 40 % (v/v) acetic acid solution. The peaks are identified as in (**A**). Three additional unidentified multimers (X, Y and Z) are also indicated. Experimental conditions were as described in [16].

invariable amounts (see Figure 2B) provided that experimental conditions are identical [20]. The amount of multimers obtained is usually about or above 26–30 % of the total protein subjected to lyophilization. The rest can be recovered as monomeric, enzymically active RNase A [15,16,20]. Recently it was found that structurally identical multimers can be formed by simply heating to various temperatures (up to 60–70 °C) highly concentrated (67–200 mg/ml) RNase A dissolved in 20 % or 40 % (v/v) aqueous ethanol or 40 % (v/v) aqueous 2,2,2-trifluoroacetic acid, as well as in other media and/or at different pHs, but always avoiding the lyophilization step [17]. However, under these conditions [17] the oligomers are formed in reciprocal proportions that are quite different from the canonical and remarkably invariable amounts obtained with the lyophilization procedure [20]. It has also been reported that concentrated RNase A undergoes a small amount of oligomerization at 23 °C [17], and that spontaneous dimerization of RNase A occurs at high substrate concentrations [33] or in buffer solutions maintained at 37 °C or 65 °C [34], which strengthens the possibility that RNase A oligomerization

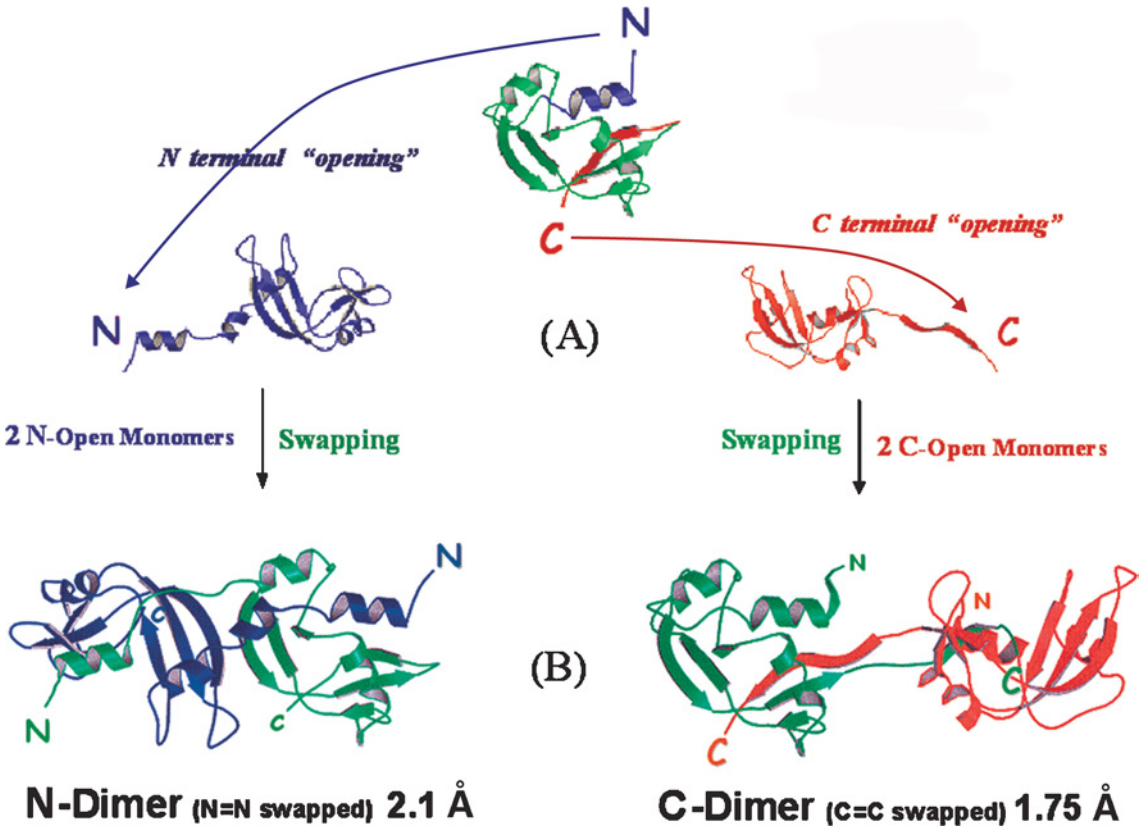

Figure 3 Structures of the two dimeric conformers of RNase A

(**A**) 'Opening' of the N-terminus or C-terminus of RNase A as the first step in the formation of 3D domain-swapped oligomers. N, N-terminal α-helix; C, C-terminal β-strand. (**B**) The two dimers of RNase A. The N-dimer (N=N swapped) is shown at 2.1 Å resolution (PDB code 1A2W). Modified, with permission, from Liu, Y., Hart, P. J., Schlunegger, M. P. and Eisenberg, D. (1998) Proc. Natl. Acad. Sci. U.S.A. **95**, 3437–3442. © (1998) National Academy of Sciences, U.S.A. The C-dimer (C=C swapped) is shown at 1.75 Å resolution (PDB code 1F0V). Modified, with permission, from Liu, Y., Gotte, G., Libonati, M. and Eisenberg, D. (2001) Nat. Struct. Biol. **8**, 211–214. © Nature Publishing Group.

is physiologically meaningful. The stability of the aggregates, which is quite good in 0.2 M sodium phosphate buffer, pH 6.7 [11,20], decreases going from dimers to the larger oligomers. All of them dissociate over time, with the larger oligomers forming intermediate, smaller oligomers before complete dissociation. The stability of RNase A oligomers depends closely on temperature, protein concentration and ionic strength [20]. Moreover, as would be expected theoretically, the stability of the RNase A oligomers increases when they form a complex with a nucleic acid. This has been demonstrated with the C-trimer and the CNC-tetramer, whose structures exposed to a temperature of 45 °C for 2.5 min were definitely stabilized by the presence of double-stranded poly(dA-dT) · poly(dA-dT) [20]. It is worth noting here that among the most important features of the RNase A oligomers are the arrangement and density of their basic charges (which of course change on going from smaller to larger oligomers), to which their biological actions appear to be related, as we shall see later.

The two RNase A dimers

As mentioned above, dimerization, or the oligomerization of RNase A generally, may occur by the swapping of only its N-terminus (residues 1–15) or C-terminus (residues 116–124) (Figure 3), or both. In each case the integrity and functionality of the active site (His-12, His-119 and Lys-41) is maintained because of its composite nature. By swapping, the N-terminal or C-terminal domains adhere to their new partner in such a way that His-12 or His-119 can find their proper location and correctly reconstitute the enzyme active site [3,11–14,18–20].

N-dimer

This is formed by the swapping of the N-termini of each monomer (PDB entry 1A2W). Figure 3 shows the minor, less basic RNase A dimer, recently named the N-dimer [17,35], for which crystals were obtained and the structure solved in 1998 by Liu, Hart, Schlunegger and Eisenberg [18]. The N-terminal helix (residues 1–15) of each monomer is swapped into the major domain (residues 23–124) of the second monomer. The two subunits of this dimer interact through their three-stranded β-sheets, forming a six-stranded sheet across the interface of the dimer. This is the new interface (the 'open interface' in the terminology introduced by Eisenberg; see [14,18,36]) between the two subunits of the dimer, and is not present in the monomer of RNase A. This structure is remarkably different from that of BS-RNase (bovine seminal RNase) [37] (see Figure 4), the only natural ribonuclease dimer known so far [38], in which the interface between the two subunits (the 'open interface') is formed by the two N-terminal α-helices (residues 24–33), and is stabilized by two disulphide bonds existing between Cys-31 and Cys-32 of each subunit. Interestingly, and with important functional consequences, there is a conformational equilibrium between the helices and the protein cores, with the helices being swapped two-thirds of the time [38].

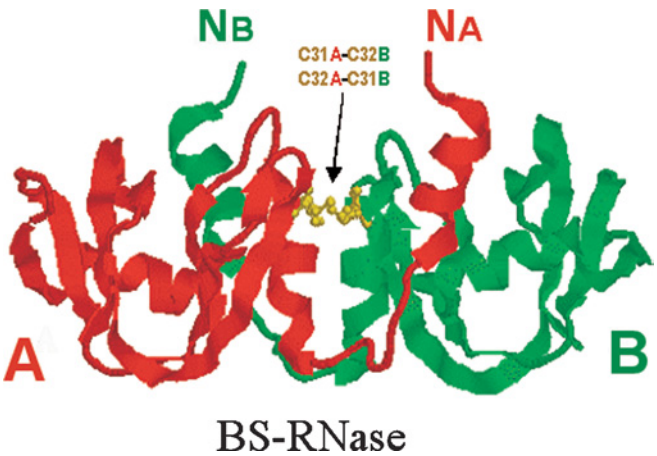

Figure 4 Structure of BS-RNase

The two disulphide bridges stabilizing the quaternary structure of BS-RNase are indicated. The N-termini of the two subunits swap in about 70% of BS-RNase molecules in solution (MxM form [38]; PDB code 1BSR), as shown in this picture.

C-dimer

The C-dimer is formed by the swapping of the C-termini of each monomer (PDB entry 1F0V). It represents the second and significantly more abundant RNase A dimer, the structure of which (Figure 3), solved in 2001 [3], is completely different from that of the N-dimer, as well as from that of dimeric BS-RNase. This dimer, previously called the major or more basic dimer [3,16] and recently renamed C-dimer [17,35], is formed by the swapping of the C-terminal β-strand of each RNase A monomer (residues 116–124), producing a molecule whose size is larger than that of the N-dimer. While its hinge loop, i.e. the segment linking the two swapping domains, is shorter (amino acids 112–115) than that of the N-dimer (residues 16–22), the total molecular surface per dimer is 13411 Å^2 for the C-dimer compared with 12236 Å^2 for the N-dimer, and its largest molecular dimension is 85 Å compared with 77 Å for the N-dimer [3]. These larger dimensions are illustrated by the fact that the C-dimer elutes from gel-filtration columns before the N-dimer (Figure 2A). The more elongated structure of the C-dimer exposes more positive charges as compared with the N-dimer (Figure 5), as clearly shown by their elution positions in the cation-exchange chromatogram of Figure 2(B).

The two RNase A trimers

NC-trimer

In this trimer, both the N-termini and C-termini of the protein swap. This oligomer, once called the major and less basic trimer [3,16], has yet not been crystallized. Its structure has therefore

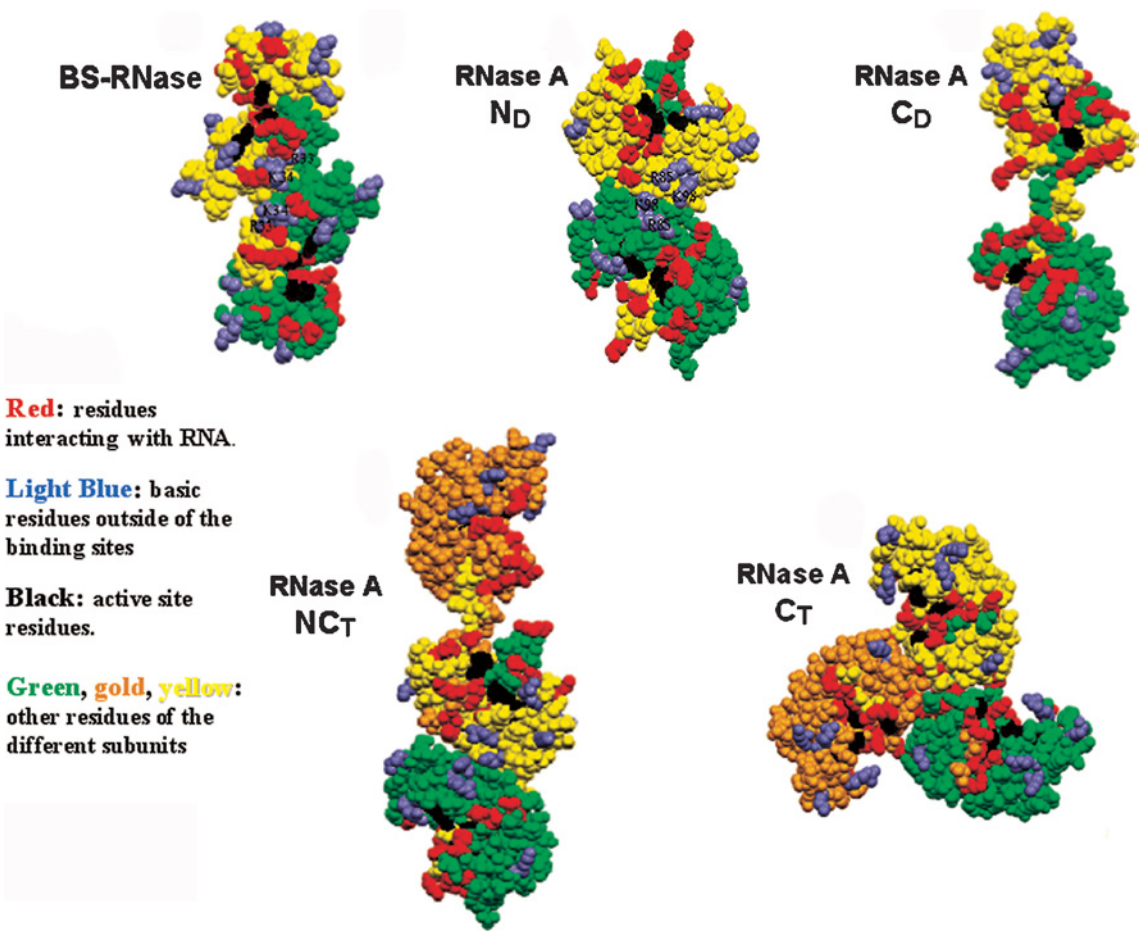

Figure 5 Distribution of positive charges on RNase A multimers and BS-RNase

N_D, N-dimer; C_D, C-dimer; NC_T, NC-trimer; C_T, C-trimer. Modified with permission from Liu, Y., Gotte, G., Libonati, M. and Eisenberg, D. (2002) Protein Science **11**, 371–380. © Cold Spring Harbor Laboratory Press.

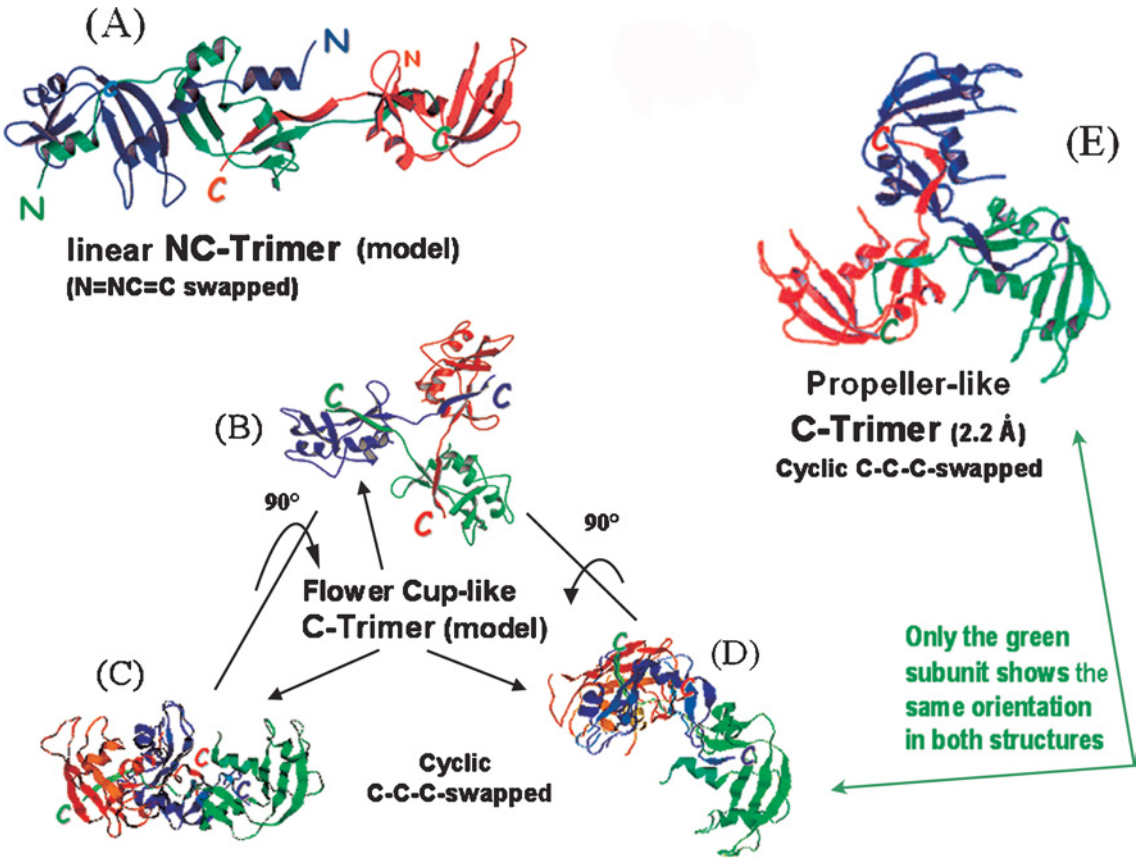

Figure 6 The two RNase A trimers

(**A**) Linear model for the RNase A NC-trimer (N=NC=C swapped). Modified with permission from Liu, Y., Gotte, G., Libonati, M. and Eisenberg, D. (2001) Nat. Struct. Biol. **8**, 211–214. © (2001) Nature Publishing Group and from Liu, Y., Gotte, G., Libonati, M. and Eisenberg, D. (2002) Protein Science **11**, 371–380. © Cold Spring Harbor Laboratory Press. (**B**–**D**) Flower cup-like model of the cyclic trimer. (**E**) Structure of the propeller-like C-trimer (C–C–C swapped) at 2.2 Å (PDB code 1JS0). Modified with permission from Liu, Y., Gotte, G., Libonati, M. and Eisenberg, D. (2002) Protein Science **11**, 371–380. © Cold Spring Harbor Laboratory Press.

been derived from data obtained by studying the spontaneous dissociation of the oligomer, its cross-linkage with 1,5-difluoro-2,4-dinitrobenzene, and its susceptibility to digestion with subtilisin [19,20]. On this basis, a linear model for the trimer, the more abundant of the two trimeric conformers, was proposed [3,19] (Figure 6A), with the two types of domain swapping contributing to its formation. Therefore it was later named the NC-trimer [35]. It can be envisaged either as an N-dimer, one subunit of which swaps its C-terminus with the C-terminus of a third monomer, or as a C-dimer swapping the N-terminus of one of its two subunits with the N-terminus of a third subunit. The linear trimeric molecule is obviously longer than both RNase A dimers. In fact, in gel-filtration experiments it elutes before the C-dimer, which in turn elutes before the N-dimer (Figure 2A). Moreover, the density of positive charges in its structure is higher than that of both dimers (see Figure 5), which explains its cation-exchange chromatographic properties. The NC-trimer is indeed retained by the resin more strongly than the two dimers, and elutes after the C-dimer in the typical ion-exchange chromatogram shown in Figure 2(B).

C-trimer

In this case each of the three monomers swaps its C-terminus (PDB entry, 1JS0). On the basis of spontaneous dissociation experiments with the less abundant trimeric conformer, indicating the formation of only the C-dimer and the RNase A monomer as intermediate products, a cyclic 'flower cup-like' model was initially constructed for this RNase A oligomer (Figures 6B–6D), with the C-terminal strands swapped and the three subunits placed with a reciprocal symmetry of 120° [19]. The model was found to be similar but not identical to the real cyclic structure of the trimer derived from X-ray analyses of its crystals [19] that is shown in Figure 6(E). The stability of the quaternary structure of the C-trimer appears to be lower than that of the NC-trimer under a particular set of defined conditions [20]. The shape of this interesting structure is rather compact and looks similar to a propeller. Oligomerization occurs by the swapping of the C-terminus of each monomer into the body of the next monomer; thus this trimer, once called the minor and more basic trimer [3,16,19], was renamed the C-trimer [35]. The composite active site of each subunit is therefore made by His-12 and Lys-41 belonging to one RNase A monomer, and His-119 belonging to another monomer. The density of positive charges of this trimer (see Figure 5) explains its elution position in the ion-exchange chromatography shown in Figure 2(B). The production of this type of oligomer indicates once more the peculiar nature of the RNase A protein, which is not only able to swap both its N-terminus and C-terminus, but is also able to form linear as well as cyclic oligomeric structures because of the high flexibility of the C-terminal hinge loop (Figures 6A–6E). In both instances RNase A appears to be a unique protein.

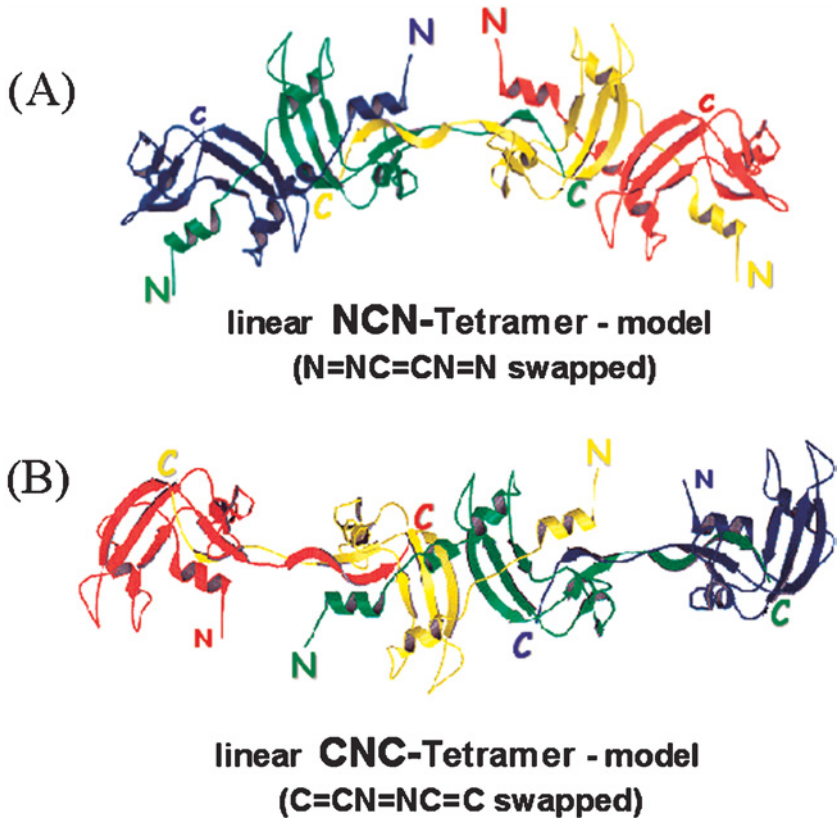

Figure 7 Linear models of the two RNase A tetramers

(**A**) NCN-tetramer (N═NC═CN═N swapped). Its structure can be envisaged as being formed by a central C-dimer, each of the two subunits of which swaps its N-terminus with that of another RNase A monomer. (**B**) CNC-tetramer (C═CN═NC═C swapped). Its structure is envisaged as being formed by a central N-dimer, each of the two subunits of which swaps its C-terminus with that of another RNase A monomer. Modified, with permission, from Liu, Y. and Eisenberg, D. (2002) Protein Science **11**, 1285–1299. © Cold Spring Harbor Laboratory Press.

RNase A tetramers

NCN-tetramer

This tetramer comprises one swapped C-terminus and two swapped N-termini. The relatively small amount of this and the next tetrameric conformer of RNase A has made their characterization difficult. However, on the basis of their dissociation kinetics, cross-linking experiments and susceptibility to digestion by subtilisin, two linear models were proposed [20], each of which involves two types of 3D domain swapping. The tetramer once called the minor or less basic tetramer [3,16] contains two N-terminal types and one (central) C-terminal type of domain swapping. On this basis it was named the NCN-tetramer [35] (Figure 7A). Its linear structure can be envisaged as being formed by a central C-dimer, each monomer of which swaps its N-terminus with the N-terminus of another monomer. The NCN-, minor tetramer has a total density of positive charges lower than that of the more abundant CNC-tetramer, and therefore shows different ion-exchange chromatographic behaviour (Figure 2B).

CNC-tetramer

The CNC-tetramer contains one swapped N-terminus and two swapped C-termini. The linear model for this second type of tetrameric RNase A, previously called the major or more basic tetramer [15,20], can be pictured as a central N-dimer whose monomers have exchanged their C-termini with the C-terminus of another RNase A monomer (Figure 7B). Since an N-terminal swapping links the central pair of monomers that swap their C-termini to join the exterior monomers, this tetramer has been named the CNC-tetramer [35]. Due to the greater exposure of positive charges characteristic of the C-dimer in comparison with the N-dimer [15,19,20] (Figure 5), the CNC-tetramer has a positive charge density higher than that of the NCN-tetramer, which explains the greater retention of the former by the cation-exchange resin (Figure 2B). Also, the CNC-tetramer is slightly larger, because of the presence of two C-dimers, than the NCN-tetramer, which accounts for the clear separation of the two oligomers on gel filtration (Figure 2A). The stability of the quaternary structure of the CNC-tetramer appears to be lower than that of the NCN-tetramer under a particular set of defined conditions [20]. However, as for the trimers (see above), this result might not be general, as the quaternary structure stability of RNase A oligomers in general, and of the tetramers in particular, is highly dependent on the environmental conditions, including buffer, pH, ionic strength and temperature [20,39].

The NCN- and the CNC-tetramers might be also respectively considered as an NC-trimer with the addition of an N-swapping monomer on one end or of a C-swapping monomer on the other end of the molecule. By adding additional monomers on the ends of either linear tetramer, long linear oligomers with alternating N- and C-swapped termini could theoretically form.

Other models of tetrameric RNase A

Two linear models for the tetramers of RNase A, identical to those described and discussed above, were also proposed by Liu and Eisenberg [14], but in addition these authors advanced two

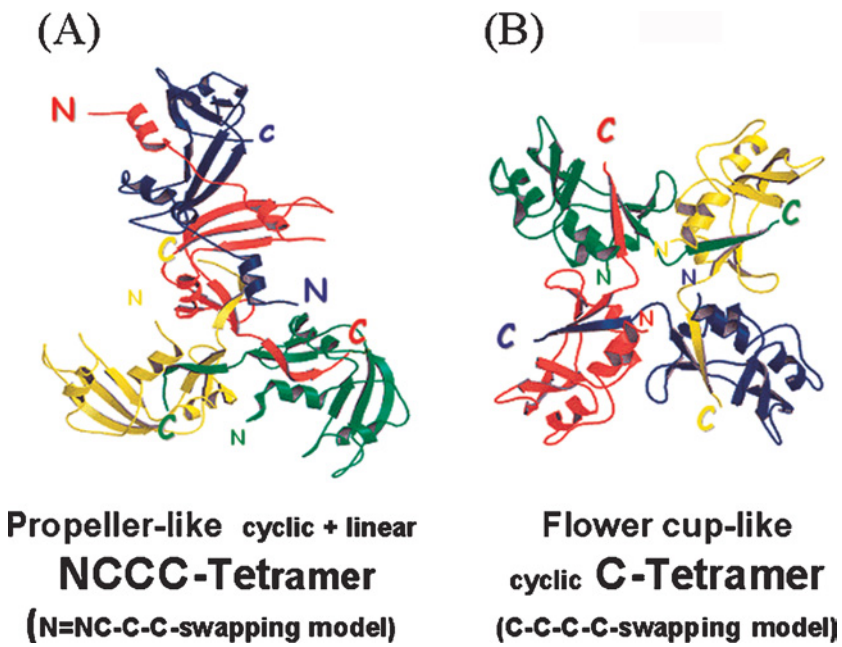

Figure 8 Models for additional possible tetrameric structures of RNase A

(**A**) Propeller-like, NCCC-tetramer (N=NC–C–C swapped). This tetramer can be envisaged as a C-trimer, one of the subunits of which swaps its N-terminus with that of an RNase A monomer. (**B**) Cyclic, flower cup-like tetramer (C–C–C–C swapped). Modified, with permission, from Liu, Y. and Eisenberg, D. (2002) Protein Science **11**, 1285–1299. © Cold Spring Harbor Laboratory Press.

additional, quite different tetrameric models [14]. One of them is a combination of a cyclic, propeller-like molecule and a linear structure, containing both types of swapping (Figure 8A). The other is the model of a cyclic flower cup-like tetramer, formed by the swapping of the C-terminus of each monomer, and with the four subunits showing a reciprocal symmetry of 90° (Figure 8B). Other cyclic or composite (cyclic plus linear) tetrameric structures could exist, and future experimental work might confirm these predictions. A final observation: cyclic trimers or tetramers could be considered as a first nucleus from which branches of linear oligomers might protrude and develop to form further, more complex structures. Moreover, the picture could be made more complicated by assuming that linear and/or cyclic oligomers with an identical type of domain swapping could have different structures because of different reciprocal orientations of their subunits (see the model and the real structure of the C-trimer; Figures 6B–6E).

RNase A pentamers

The existence of these higher-order multimers has been deduced from the presence of two small protein peaks eluting after the two tetramers, at a higher salt concentration, in cation-exchange chromatograms (Figure 2B), and of two discrete, unique bands appearing in cation gel electrophoresis analyses performed under non-denaturing conditions [16]. Moreover, a third piece of evidence is a peak (P) eluting before the two tetramers in the gel filtration chromatogram shown in Figure 2(A), that could reasonably be interpreted as a pentameric RNase A species. Theoretically, there could be several pentamers of RNase A. Their quaternary structures can be imagined on the basis of the linear or composite oligomers described above (see Figures 7 and 8).

The first of the two putative pentameric species eluted from the ion-exchange chromatographic column (P in Figure 2B) was assayed for its enzyme activity on dsRNAs, and was shown to be endowed with the highest depolymerizing activity measured [16]. This is consistent (see below) with the charge properties of an RNase A pentamer. However, the difficulty in obtaining these higher-order oligomers in sufficient amounts for their characterization does not allow us to describe them in more detail.

Implications of the C-dimer structure for amyloid formation

One point deserves further comment; namely, the implications of the C-dimer structure for amyloid formation [3]. This has to be regarded as one facet of the general hypothesis [3,18,40,41] that proposes the involvement of 3D domain swapping in the production of the large aggregates of cross-β structure that have been found in neurons of people suffering from many neurodegenerative diseases, and that therefore have been linked to the development of these diseases.

Although the solubility and functional properties of all RNase A oligomers, and the C-dimer in particular, differentiate them from insoluble protein aggregates in general, and especially from amyloid, some interesting structural details highlight the utility of the RNase A multimers as models for interpreting the features of pathological aggregates. Moreover, the recent finding that early aggregates of native non-disease-associated proteins can become inherently highly cytotoxic [42] greatly increases interest in the first event(s) that cause protein aggregation and in the mechanism(s) that adjust the formation of these early aggregates. 3D domain swapping, a mechanism for oligomer assembly [36], has also been proposed as a mechanism for amyloid formation, since oligomer assembly and amyloid formation share common features: both events are characterized by high specificity; both result in two stable forms, separated by a high energy barrier; and both can lead to the formation of linear aggregates [14]. The idea that every protein, provided that its concentration is high and the environmental conditions are such as to partially destabilize its native structure, can aggregate to form amyloid fibrils [4] could be extended by saying that every protein under the conditions mentioned above can oligomerize by 3D domain swapping [3].

With regard to the C-dimer of RNase A, the pattern of hydrogen bonding observed in the interface (the 'open interface') between its two hinge loop regions [3] is reminiscent of the polyglutamine aggregates characteristic of the protein huntingtin, which assembles into the amyloid fibrils found in people afflicted with Huntington's disease [2,43]. This RNase A hinge loop forms a two-stranded antiparallel β-sheet. Moreover, in the C-dimer the two main-chain groups of an Asn residue located at position 113 in each of the two strands of the hinge loop (residues 112–115) form two hydrogen bonds, as occurs in antiparallel β-sheets. However, the side chain of Asn-113 also forms a third hydrogen bond with the side chain of the Asn residue of the opposite strand [3]. This pattern of hydrogen bonding is similar to the minimal unit of the antiparallel β-sheet 'polar zipper' model proposed by Perutz et al. [2,43] to stabilize the polyglutamine structures in the aggregates of huntingtin. By combining the observations of 3D domain swapping and polar zipper hydrogen bonding in the C-dimer of RNase A, a '3D domain swapping zipper model' for protein fibre formation was proposed [3]. Without going into structural details here, we refer the reader to the description and discussion of this model [3], just mentioning that it can also account for electron microscopic results with other amyloidogenic proteins.

It is worth noting that the hypothesis that 3D domain swapping is involved in amyloid formation is supported by recent findings that the amyloidogenic protein cystatin C [44], and the human prion protein [45], form 3D domain-swapped dimers.

Factors affecting ribonuclease oligomerization

Modification of the protein – glycosylation and deamidation

What factors could sterically influence RNase A oligomerization? In particular, could the carbohydrate moiety of a glycosylated RNase affect the ability of the protein to oligomerize? This question was addressed by subjecting bovine RNase B to the aggregation procedures described for RNase A [46]. RNase B is the glycosylated version of RNase A, the only difference between the two proteins being the presence in the former of a single oligosaccharide chain, consisting of a common trunk of two *N*-acetylglucosamine residues and between five and nine mannose residues, attached to Asn-34. Experimental evidence has been obtained that the presence of this rather small carbohydrate chain at position 34 of RNase B enhances both the rate of formation and the amount of the N-dimer, while reducing the production of the C-dimer. This result is surprising, since the carbohydrate chain is positioned near enough to the N-terminus to sterically hinder its swapping, but is relatively far from the C-terminus. A rather naive explanation could of course be that under the conditions and during the course of the oligomerization process the flexible carbohydrate chain might shift towards the C-terminus of the ribonuclease molecule, therefore inhibiting the formation of the C-dimer. A second possible explanation is to postulate the presence of stabilizing interactions between the carbohydrate chains of different monomers, with each other or with the other protein moiety. Such hypothetical interactions could also account for the experimental observation [46] that RNase B oligomers form more rapidly than those of RNase A.

RNase B, like RNase A, contains a single Asn-67–Gly-68 residue pair that undergoes selective deamidation [46], as also occurs in RNase A [47]. An additional experimental finding was that this modification decreased the 3D domain-swapping event, and therefore the oligomerization of the protein [46]. This is probably due to a steric modification of the protein loop containing Asn-67 and Gly-68 that subsequently affects the manner in which this sterically modified loop interacts with the C-terminus of the protein [48].

Different amino acid compositions of the RNase A N-terminus and C-terminus

Another factor capable of influencing the aggregation of RNase A could be the different amino acid compositions of the two regions of the protein involved in its oligomerization through 3D domain swapping, i.e. the N-terminus and the C-terminus.

The two termini of RNase A differ: the helical N-terminus is relatively richer in hydrophilic amino acids, whereas the C-terminus has a higher proportion of hydrophobic residues and forms a β-strand. According to Chiti et al. [8], the regions of a protein capable of inducing its aggregation are those that are relatively rich in hydrophobic residues and prone to form β-sheet structures. Therefore, under conditions where both termini of RNase A are unfolded, the aggregation process should be favoured at the C-terminus. Experimental results obtained with the thermal treatment method (without the lyophilization step) [17] are in agreement with this hypothesis. They indicated that under vigorous denaturing conditions, where both termini are presumably unfolded, formation of the C-dimer prevails over formation of the N-dimer. In contrast, under milder denaturing conditions, where the unfolding of the N-terminus presumably exceeds that of the C-terminus due to the weaker attachment of the former to the protein core, the formation of the N-dimer prevails over that of the C-dimer. The interpretation of the experimental results obtained [17] as ascribable to the different amino acid compositions of the N- and C-termini of the RNase A molecule could be tested by modifying their polar or apolar character by site-directed mutagenesis, and then subjecting the mutants to the same unfolding and aggregating conditions used with the wild-type protein. Such studies could aid our understanding of the factors controlling the 3D domain-swapping mechanism that leads to the aggregation of proteins in general, and to the oligomerization of RNase A in particular.

FUNCTIONAL ASPECTS OF RNase A MULTIMERS

Enzymic activity

All of the RNase A oligomers isolated so far that have a composite active site are enzymically active. As mentioned above, oligomerization, induced either by the lyophilization procedure [11] or by the alternative methods that avoid the lyophilization step [17], reconstitutes the active site disrupted by the partial unfolding of the protein. The 3D domain-swapping mechanism, by which each oligomer is formed, permits the reconstitution of the spatial arrangement characteristic of the active site of RNase A, as was originally envisaged and demonstrated by Crestfield, Stein and Moore [11], and Fruchter and Crestfield [13]. His-12 is contributed by the swapping of the N-terminus (residues 1–15), and His-119 by the swapping of the C-terminus (residues 116–124), while Lys-41 is in the core of the protein. Based on their assays of the enzyme activity of monomeric RNase A and its dimers using 2′,3′-cyclic cytidylate as substrate, Crestfield and coworkers [11–13] stated that there was no difference in specific activities between monomeric RNase A and its aggregates. A slightly different result was obtained by using single-stranded (yeast) RNA or synthetic polyribonucleotides as substrates [15,16,49], and a completely different result was found with dsRNAs or poly(A) as substrates [15,16,29,30,49,50].

ssRNAs as substrates

With yeast RNA as substrate, and measuring the enzyme activity using the spectrophotometric procedure of Kunitz [51], the specific activity of commercial (Sigma; type XII-A) bovine monomeric RNase A is approx. 100 Kunitz units/mg. The oligomers

of RNase A show between 40 % and 80 % of the specific activity of the RNase A monomer, with the larger oligomers generally having lower activity values than the smaller oligomers [15,16,49]. With polyuridylate or polycytidylate as substrates, the oligomers' decrease in specific activity is less, with values of between 70 % and 90 % of that determined for monomeric RNase A [15,16]. It has to be taken into account that, while activities expressed per mg of oligomeric protein decrease progressively as the size of the oligomers increases, the activities expressed per mol of enzyme species are all approx. 60 % of the activity of monomeric RNase A. To explain these data, we proposed that probably not all of the active sites of the larger oligomers are available simultaneously for interaction with a ssRNA. This would lead, therefore, to decreased catalytic efficiency as a function of the increasing size of the oligomers. Whereas, for example, four distinct molecules of monomeric RNase A could interact successfully with a single-stranded substrate, each one cleaving one phosphodiester bond, we envisage that not all four active sites of a tetrameric RNase A molecule, but just a fraction of them, could interact productively with the single-stranded substrate. If this were the case, the overall depolymerizing activity displayed by four distinct monomeric RNase A molecules would obviously be higher than that displayed by a tetrameric RNase A molecule, supporting the results obtained [15,16]. On the other hand, some of the other three 'silent' active sites present in a tetramer could be or become available and interact with the ssRNA, producing additional activity. This activity could compensate for the actual number of molecules of the oligomer, which, in the unit of solution volume, decreases progressively as the size of the oligomers increases. The compensatory activity could be estimated by expressing the enzymic activity not per mg but per mol of oligomeric species. It should be also taken into account that the reduced activity could perhaps be due to a kind of product inhibition: the ssRNA may stay fixed in place on the larger oligomers due to their higher positive charge, making diffusing to a position suitable for cleavage more difficult than for the ssRNA bound to the surface of an RNase A monomer.

It is difficult to explain why homopolymers such as polyuridylate or polycytidylate are slightly better substrates than yeast RNA. However, the length of poly(U) or poly(C) molecules (which is presumably higher than that of commercial yeast RNA), and in particular their homogeneity (being composed, unlike yeast RNA, of only pyrimidine bases) may allow better binding of the polyribonucleotides to the RNase A subsites (B1, formed by Thr-45 and Asp-83, selective for pyrimidine bases, in particular cytosine; B2, formed by Asn-71 and Glu-111, which binds all bases, but prefers adenine; B3, whose existence was inferred from kinetic and chemical data, which prefers purines), therefore explaining their greater suitability as single-stranded substrates [23,52,53]. Moreover, yeast RNA, because of its mixed sequence, folds into a more complicated 3D structure than do homopolymeric sequences. This may also help to explain the higher reactivity of poly(U) or poly(C) in comparison with yeast RNA.

dsRNAs as substrates

Completely different results are obtained when the substrate is a synthetic double-stranded polyribonucleotide such as poly(A) · poly(U) or authentic viral dsRNA. As mentioned above, single-stranded homopolyribonucleotides, such as poly(U) or poly(C), are degraded very efficiently by RNase A, possibly because of their fruitful binding to all available subsites of the enzyme [23,52,53]. In contrast, dsRNA is highly resistant to digestion by RNase A because the stereochemical requirements for the 'in line' mechanism of action of the enzyme do not allow a productive interaction between its active site and the double-helical RNA [28]. It might be worth recalling here that the experimental conditions under which a ribonuclease assay against dsRNA is carried out have to be chosen carefully in order to guarantee the integrity and stability of the secondary structure of the nucleic acid, and also to take into account the rather complex influence of the salt concentration on ribonuclease activity against dsRNA [29,30]. A classic medium is a buffered (pH 7.0) solution of 0.15 M NaCl containing 0.015 M sodium citrate [29,30,54], but any other buffer at neutral pH with an ionic strength of approx. 0.15 can be convenient. However, the resistance of dsRNA to attack by RNase A is never absolute, because thermal fluctuations of the nucleic acid secondary structure continually produce transitory breaks in the hydrogen bonds between the two strands, and thus decrease the compactness and integrity of dsRNA, particularly at the ends of the RNA double helix, therefore allowing sporadic cleavage by the enzyme.

Whereas monomeric RNase A is 'inactive' against dsRNAs, its multimers are definitely active, and this activity increases with the size of the oligomer [15,16,19,49]; that is, trimers are more active than dimers, tetramers are more active than trimers, and pentamers are more active than tetramers. Moreover, within each pair of conformers, the one endowed with a higher 'density' of positive charges shows the higher activity [16] [Tables 1 and 2; in Table 1 the results obtained with poly(A) · poly(U) as substrate are qualitatively better than those obtained with viral dsRNA, but all of them are consistent with the results of many other individual assays not considered here]. In other words, the C-dimer is more active than the N-dimer, the C-trimer is more active than the NC-trimer, and the CNC-tetramer is more active than the NCN-tetramer.

This behaviour has been related to the 'basicity' of the oligomer molecule, i.e. to a hypothesis already advanced to explain the remarkable activity towards dsRNA shown by several mammalian pancreatic-type monomeric ribonucleases, as well as by the naturally dimeric BS-RNase [29,30,55–57]. All of these RNases share the property of having a higher number of positively charged

Table 1 Enzyme activity of bovine RNase A multimers against double- and single-stranded polyribonucleotides

The incubation mixture comprised 0.44 ml of 0.136 M NaCl, 0.0136 M sodium citrate and 0.016 M sodium phosphate buffer, pH 6.7, containing 45 μg of poly(A) · poly(U) or 35 μg of f2 *sus11* bacteriophage dsRNA. Enzyme concentrations were as follows: RNase A monomer, 1.6 mM; N-dimer (N_D) and C-dimer (C_D), 0.33 mM with both poly(A) · poly(U) and viral dsRNA as substrates; NC-trimer (NC_T) and C-trimer (C_T), 0.13 mM; NCN-tetramer (NCN_{TT}) and CNC-tetramer (CNC_{TT}), 0.066 mM; putative pentamer (P), 0.039 mM with poly(A) · poly(U), but 10× less concentrated with viral dsRNA. The assay was performed at 22 °C, and calculations of enzyme activity and specific activity were performed as described in [16,30]. Modified, with permission, from Gotte, G., Bertoldi, M. and Libonati, M. (1999) Eur. J. Biochem. **265**, 680–687. © FEBS 2002 and Blackwell Publishing.

RNase A species	Specific activity (activity per nmol of enzyme species)				
	Poly(A) · poly(U)	Viral dsRNA	Poly(A)*	Poly(C)	Yeast RNA
Monomer	0.15 ± 0.03	0.44 ± 0.02	0.7 ± 0.03	933.3 ± 37.1	31.9 ± 1.9
N_D	0.33 ± 0.05	1.43 ± 0.19	3.8 ± 0.16	533.0 ± 131.5	16.5 ± 2.1
C_D	2.21 ± 0.01	1.37 ± 0.19	3.7 ± 0.30	–	15.3 ± 2.1
NC_T	2.21 ± 0.02	5.00 ± 0.24	5.5 ± 0.10	721.3 ± 116.1	13.7 ± 1.7
C_T	3.74 ± 0.17	5.52 ± 0.27	7.7 ± 0.08	606.5 ± 250.8	11.7 ± 1.6
NCN_{TT}	4.79 ± 0.58	11.72 ± 0.23	10.3 ± 0.55	804.3 ± 46.4	16.0 ± 1.7
CNC_{TT}	9.52 ± 0.27	10.86 ± 0.71	10.4 ± 0.33	674.0 ± 125.0	13.7 ± 0.8
P	20.47 ± 1.11	18.86 ± 0.29	17.1 ± 0.96	833.3 ± 96.9	18.7 ± 1.0

* Specific activity $\times 10^{-3}$.

Table 2 Structure–activity correlation of various RNase A multimers for degradation of dsRNA

The incubation mixture comprised 0.4 ml of 0.15 M NaCl/0.015 M sodium citrate, pH 7, containing 40 μg/ml poly(A) · poly(U) plus 25 μg/ml RNase A monomer, 10 μg/ml N-dimer (N_D), or 5 μg/ml C-dimer (C_D), NC-trimer (NC_T) or C-trimer (C_T). The distance between the active sites was measured as described in [19]. Calculations of enzyme activity and specific activity were performed as described in [30,55]. Enzyme assays were carried out at 25 °C. Modified with permission from Liu, Y., Gotte, G., Libonati, M. and Eisenberg, D. (2002) Protein Science **11**, 371–380. © Cold Spring Harbor Laboratory Press.

RNase A species	Distance between active sites (Å)	Specific activity against poly(A) · poly(U) (units/mg of protein)
Monomer	–	2.7 ± 0.5
N_D	39.6	4.5 ± 0.8
C_D	38.7	30 ± 3
NC_T	39.6 and 38.7	32 ± 6
C_T	28.8	61 ± 5

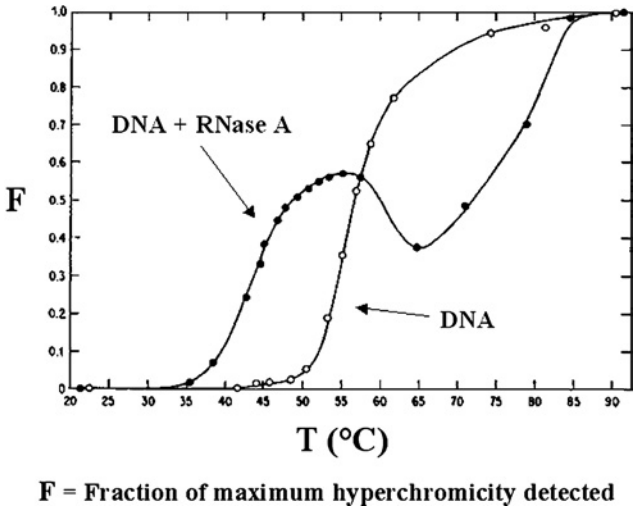

Figure 9 DNA-helix-unwinding activity of RNase A

Thermal-transition profiles of calf thymus DNA in the presence and absence of bovine RNase A. Approx. 33 μg/ml DNA was mixed with 170 μg of RNase A in 0.002 M NaCl/0.001 M phosphate, pH 7.1. Modified with permission from Felsenfeld, G., Sandeen, G. and von Hippel, P. H. (1963) Proc. Natl. Acad. Sci. U.S.A. **50**, 644–651. © Dr Gary Felsenfeld.

amino acids located specifically in the region of their active site when compared with bovine RNase A [30,55]. Their degrading action towards dsRNAs sometimes reaches very high values, as occurs with human pancreatic ribonuclease [30,55,58,59]. Other RNases, such as human non-pancreatic-type ribonucleases (e.g. RNases 2 and 3), are highly basic proteins, but have many of their basic amino acids located far away from the active-site region, and are totally inactive towards double-stranded poly(A) · poly(U) [30,55,60,61]. They can, therefore, be considered as genuine 'single-strand-specific' ribonucleases, whereas RNase A is the prototype of 'single-strand-preferring ribonucleases' [30,55].

The relationship between the number and location of positive charges on a ribonuclease molecule and its activity against dsRNA found a basis in the observation published by Felsenfeld, Sandeen and von Hippel in 1963 on the DNA-helix-unwinding ability of bovine pancreatic RNase A [62] that is described in the next paragraph.

DNA-helix-unwinding ability of oligomeric RNase A, and its relationship with the degrading activity of RNase A oligomers towards double-helical RNA

The experiment performed by Felsenfeld et al. [62] showed that RNase A is an efficient 'destabilizer' of the secondary structure of double-stranded DNA. The thermal transition profile of the complex formed by calf thymus DNA and RNase A in 0.002 M NaCl/0.001 M phosphate buffer, pH 7.1, revealed that the T_m of the nucleic acid measured in the presence of RNase A was approx. 18 °C lower than that determined in its absence (Figure 9). BS-RNase [57], as well as various mammalian monomeric pancreatic-type ribonucleases (but not non-pancreatic-type RNases [55]), were also later found to have this destabilizing action [56,63,64], which proved to increase as a function of the number and specific location of the positive charges present on these molecules [29,30,55,65].

A qualitatively similar helix-unwinding activity was observed for double-stranded poly(dA-dT) · poly(dA-dT) mixed with the RNase A C-dimer, C-trimer or CNC-tetramer in a buffer whose ionic strength was significantly higher [20] than that employed by Felsenfeld et al. [62]. The thermal transition profiles of these mixtures indicated clear destabilization of the secondary structure of the nucleic acid, with a shift of the hyperchromicity values towards lower temperatures, and their increase as a function of the size (and therefore the density of positive charges) of the oligomers [20].

With the aim of explaining the mechanism of degradation of dsRNA by the various mammalian ribonucleases that are more 'basic' than RNase A, the observations concerning DNA–RNase interactions [56,57,63] were extrapolated to dsRNA–RNase interactions. At first, the idea was expressed that a prior event in the degradation process could be destabilization of the secondary structure of dsRNA by its interaction with a ribonuclease molecule [57,66,67]; the immediately subsequent step would be degradation of the destabilized RNA. Later, the helix-unwinding event was interpreted [29,30,55] in terms of the model proposed by Jensen and von Hippel [68] to explain the destabilization of DNA by RNase A: rather than inducing the helix unwinding of DNA, RNase A, because of its higher affinity for single-stranded compared with double-stranded nucleic acid structures, would trap single-stranded DNA sequences transiently exposed by the continuous thermal fluctuation of the nucleic acid secondary structure. This model can be extrapolated to dsRNA: rather than directly inducing the local helix unwinding of dsRNA, a ribonuclease molecule would rapidly bind and digest single-stranded sequences of dsRNA produced by thermal fluctuation of the nucleic acid secondary structure or, alternatively, even single nucleotides 'wound off' the double helix [29,30,69–72]. Binding and digestion efficiency would depend on the number of positive charges specifically located on the enzyme molecule. For RNase A, these events would therefore be more frequent for the larger oligomers.

This model, which has been verified several times in the past [30], has been recently confirmed and demonstrated by Sorrentino et al. [59] in experiments performed with human pancreatic RNase mutants.

Activity of oligomeric RNase A on polyadenylate

In contrast with polycytidylate and polyuridylate, polyadenylate is not a suitable substrate for RNase A, although under conditions of high enzyme and substrate concentrations, degradation of poly(A) by RNase A was reported by Beers many years ago [73]. The molecular explanation for the quite modest activity of RNase A and related pancreatic-type enzymes on poly(A) is probably that purine bases can bind only in the B2 and B3 enzyme subsites; the

B1 subsite contains Thr-45, which sterically hinders the access of purine bases, making this subsite specific for pyrimidine bases [23]. In fact, the substitution of Thr-45 by Gly or Ala removes this steric barrier and increases the activity of RNase A against poly(A) [74]. RNase A dimers and higher-order oligomers also display a higher activity against the neutral form of poly(A) than monomeric RNase A, and this activity increases as a function of the size of the oligomers and, therefore, the positive electrostatic potential on their surface [15,16,75]. This result could be partly due to the increased electrostatic interaction between substrate and oligomeric enzyme, but also to a mechanism similar to that in play when oligomers of RNase A destabilize poly(dA) · poly(dT) [20] and, by extrapolation, dsRNA. In fact, at neutral pH an ordered structure is present in polyadenylate [76,77], that would be resistant to monomeric RNase A, but could be disrupted by the RNase A multimers. {At acidic pH values (below the pK_a of adenine), poly(A) assumes a well defined and particularly stable double-stranded structure, the stability of which is increased by decreasing the concentration of salt, in contrast with what occurs with double-helical polynucleotides: when an ordered structure, like that of poly(A), depends on protonation of the bases, the dependence of T_m on ionic strength is indeed reversed. This can be ascribed to reduced favourable electrostatic interactions between protonated bases and negatively charged phosphate groups as the result of the screening of the phosphate charges. Moreover, due to effects of electrostatic interaction, protonation of bases is usually suppressed by salt [77].} The fact that various mammalian pancreatic-type RNases, included BS-RNase, all of which are more 'basic' than RNase A, are substantially more active than monomeric RNase A on poly(A) [29,30,55] is in line with this explanation. It is notable that human non-pancreatic-type RNases, which are highly basic proteins but which have most of their positive charges located not at the active-site region, show no detectable activity against polyadenylate [29,30,55], just as they lack activity against dsRNA.

Does the dimeric structure of a ribonuclease play a role in its enzymic action on dsRNAs or poly(A)?

The possible importance of the dimeric structure of a ribonuclease for its action against double-helical polyribonucleotides was considered many years ago in an attempt to interpret the action of the naturally dimeric BS-RNase on dsRNAs: it was postulated that the two strands of double-helical RNA could be degraded simultaneously by the enzyme translocating with its two active sites along the double helix [50,78,79]. However, in the light of results obtained later [29,30,55–57], this hypothesis was discarded.

Similar ideas concerning the activity of BS-RNase against dsRNA were suggested by Opitz et al. [80]. However, it must be recalled and pointed out here that all other ribonucleases found to be active at degrading dsRNA are monomeric molecules. All of these enzymes, BS-RNase included, are endowed with a higher number of positive charges than bovine RNase A [29,30], which can explain their action against dsRNA, in agreement with the interpretation given above.

The proposed central role of the positive charges present on a ribonuclease molecule may be supported by some further observations. First, RNase A oligomerized by cross-linking with dimethyl suberimidate in the form of dimers [81–83] and trimers [84] acquires the ability to degrade dsRNA, but the level of this activity is lower than that of the aggregated species [84]. This can be ascribed to the loss of some basic charges upon reaction of the bifunctional cross-linking agent with the ε-NH_2 group of lysine residues [81]. Secondly, Wang and Moore [85] attached (by cross-linking with dimethyl suberimidate) eight spermine residues to RNase A, producing a very basic derivative (polyspermine-RNase), whose action against poly(A) · poly(U) and poly(I) · poly(C) was 115 and 176 times higher respectively than that of unmodified RNase A. Polyspermine-RNase A also degraded the hybrid molecule poly(dA):poly(rU) 382 times more rapidly than did RNase A.

Finally, a role for the dimeric structure of a ribonuclease in its action against dsRNAs is doubtful even for BS-RNase, given that a reduced and alkylated (with iodoacetate) monomeric derivative of BS-RNase was found to maintain 70 % of the activity measured with the native, dimeric enzyme [86], and that this value increased to 92 % when the alkylation was performed with iodoacetamide [87]. The use of this reagent avoids adding two negative charges to the enzyme molecule, as occurs when iodoacetate is used. In conclusion, from what we have described and discussed above, a rule emerges that has been verified repeatedly in the past [29,30,55] and confirmed recently [59]: the higher the number of basic amino acids located at the active-site region of an RNase molecule, the higher its activity in degrading dsRNA.

Liu et al. [19] have shown recently that a relationship exists between some structural features present in the two RNase A dimers and in dimeric BS-RNase and the action of these enzyme species on dsRNA. Table 2 shows that the extent of dsRNA degradation increases going from the N-dimer to the C-dimer to BS-RNase. The first of the structural features pointed out by Liu et al. [19] concerns the two patches formed by the multiple binding subsites from each subunit of the dimeric molecules. They are close to each other in all of the three dimers considered, forming a binding site for dsRNA, and thus increase the affinity of binding of the three enzyme species to the nucleic acid. Moreover, Arg-85 and Lys-98 of the N-dimer, and Arg-33 and Lys-34 of BS-RNase, are located between the two binding patches (Figure 5), and can serve as additional binding subsites for dsRNA. The second feature concerns the distance between the active sites in all dimeric structures examined, and its relationship to the enzymic activity (see Table 2). Measurement of these distances clearly shows that the activity against dsRNA increases as the distance between the active sites decreases. The third structural feature affecting the ability to cleave dsRNA is the relative orientation of the two binding patches in the dimers: they are twisted around the molecules of the C-dimer and of BS-RNase, the two more active 'dimers', but are located on the same side in the N-dimer, which is the least active among the three dimeric species examined [16,19].

Briefly, the degrading activity towards dsRNA displayed by the three dimeric molecules examined increases (a) as more positive charges are located and available around the active sites; (b) as the distance between the two active sites of the dimer decreases; and (c) as the relative orientation of the two binding patches in the ribonucleases becomes more twisted around the molecules. Two (a and b) of these structural features can be related to the 'basicity' or, rather, the density of positive charges located specifically on the enzyme molecules. The third (c) can be related to the nucleic acid helix-unwinding activity displayed by the RNase A oligomers, which is also strictly linked to their surface positive electrostatic potential. These structural features also hold for the C-trimer of RNase A.

In conclusion, these observations, rather than indicating a specific relationship between the dimeric structure of a ribonuclease and its activity against dsRNA, strengthen, in our opinion, the hypothesis concerning the importance of the presence and specific locations of positive charges on a ribonuclease molecule for its action against dsRNA, and the general interpretation advanced to explain the mechanism of this action [29,30,55].

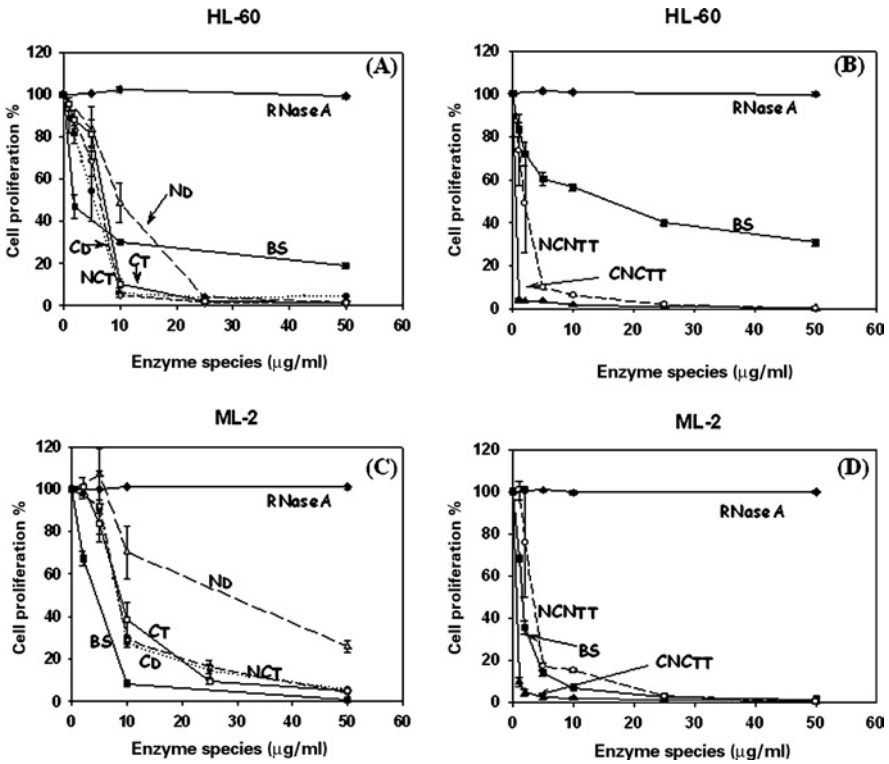

Figure 10 ***In vitro*** **antitumour activity of RNase A oligomers and BS-RNase**

(**A**) Action of the two dimers and the two trimers of RNase A (N_D, N-dimer; C_D, C-dimer; NC_T, NC-trimer; C_T, C-trimer), in comparison with that of BS-RNase (BS) and monomeric RNase A, on the proliferation of HL-60 cells, a human myeloid cell line. (**B**) Action of the two RNase A tetramers (NCN_{TT}, NCN-tetramer; CNC_{TT}, CNC-tetramer), in comparison with that of BS-RNase and monomeric RNase A, on the proliferation of HL-60 cells. (**C**) Action of the two dimers and the two trimers of RNase A, in comparison with that of BS-RNase and monomeric RNase A, on the proliferation of ML-2 cells (human myeloid leukaemia). (**D**) Action of the two RNase A tetramers, in comparison with that of monomeric RNase A and BS-RNase, on ML-2 cell proliferation. Modified with permission from Matousek, J., Gotte, G., Pouckova, P., Soucek, J., Slavik, T., Vottariello, F. and Libonati, M. (2003) J. Biol. Chem. **278**, 23817–23822. The experimental conditions are described in Matousek et al. [35].

Biological activities of the multimers of RNase A

The discovery that several members of the animal ribonuclease superfamily have multiple biological actions beyond their typical catalytic activities is rather recent. We refer the interested reader to the many excellent reviews and articles [88–96] published on this subject. Onconase, a monomeric active ribonuclease from the oocytes of *Rana pipiens* [91,97–99], and BS-RNase are well known examples of ribonucleases endowed with selectivity in their action against malignant cells [91]. Onconase is both a cytotoxin [100] and a cytotoxic agent that arrests the cell cycle in G1 phase [101]. Its LD_{50} for cytotoxicity *in vitro* is approx. 10^{-7} M, but this depends on the type of cancer cell. The protein has been tested in phase I and phase II human clinical trials for treating various solid tumours, such as lung or pancreatic cancers [102,103]. A number of studies have been performed on the antitumour, as well as the aspermatogenic, immunosuppressive and embryotoxic, activities of BS-RNase [90,91]. Its cytotoxic action was tested on malignant SVT2–3T3 fibroblasts by measuring cell survival after 48 h of growth in the presence of the enzyme, and the LC_{50} (protein concentration that kills 50 % of cells) was approx. 25 μg/ml. The cytotoxic action was selective for malignant cells, and BS-RNase was devoid of any toxicity when assayed on non-malignant 3T3 fibroblasts [104]. The same was true for cross-linked trimers of RNase A [84] (G. Gotte, L. Testolin, C. Costanzo, S. Sorrentino, U. Armato and M. Libonati, unpublished work).

Here we will mainly consider the antitumour action and some other biological properties of the oligomers of bovine RNase A, that are not found with native, monomeric RNase A. This last assertion appears to be only partly true, however, since while native RNase A is inactive if assayed at relatively low concentrations, if used in very large amounts it shows a cytotoxic activity against malignant cells. Almost 50 years ago, L. Ledoux [105,106] demonstrated that bovine RNase A used at very high concentrations was endowed with antitumour activity against Ehrlich's and Krebs's carcinomas in mice, and Walker's carcinoma in rats. This was the first time that a ribonuclease was shown to have an antitumour action.

Antitumour activity of RNase A oligomers

The absence (in the terms expressed above) of any detectable biological action of monomeric RNase A is in stark contrast with the remarkable antitumour activity of the naturally dimeric BS-RNase [91,95,107,108]. This difference, and the fact that BS-RNase loses its antitumour action, but not its enzymic activity, when it is artificially monomerized [108], might reasonably suggest that RNase A in oligomeric form may acquire antitumour activity. This was confirmed by testing the *in vivo* and/or *in vitro* antitumour action of cross-linked dimers of bovine RNase A many years ago [82,83], and of cross-linked trimers more recently [84]. Moreover, RNase A that was dimerized by site-directed mutagenesis also showed antitumour activity [109]. Upon obtaining purified conformational isomers of RNase A dimers, trimers and tetramers [16], the question arose of whether these non-covalently linked (and therefore not stabilized) dimers, trimers and tetramers of RNase A might also display some biological action.

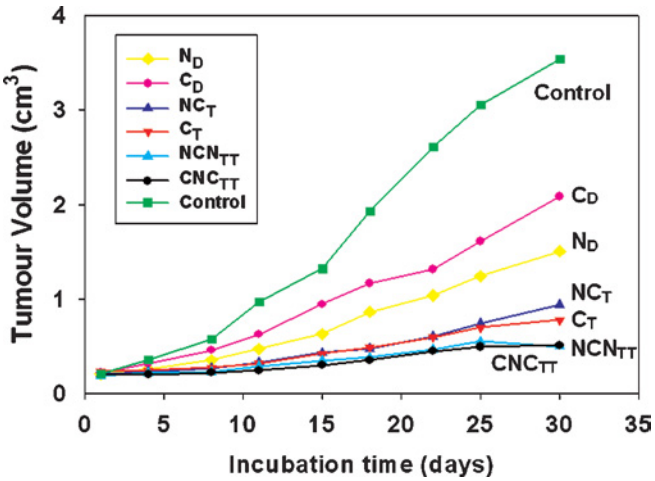

Figure 11 ***In vivo*** **antitumour activity of the various RNase A multimers**

Human non-pigmented melanoma was stabilized in tissue culture and transplanted subcutaneously in six groups of six nude mice each, plus one series of 11 mice as controls. Each inhibition value is the mean of six measurements. RNase A oligomers (250 μg/20 g) were injected intravenously three times per week for 4 weeks. Saline solution was administered to control mice. The percentage tumour growth inhibition was calculated as 1 − (mean tumour volume in treated group/mean tumour volume in controls) × 100. N_D, N-dimer; C_D, C-dimer; NC_T, NC-trimer; C_T, C-trimer; NCN_{TT}, NCN-tetramer; CNC_{TT}, CNC-tetramer. Modified with permission from Matousek, J., Gotte, G., Pouckova, P., Soucek, J., Slavik, T., Vottariello, F. and Libonati, M. (2003) J. Biol. Chem. **278**, 23817–23822. © The American Society for Biochemistry and Molecular Biology.

Experiments performed *in vitro* and *in vivo* (Figures 10 and 11) with RNase A multimers obtained by the lyophilization procedure of Crestfield et al. [11] have shown that all oligomers are indeed endowed with antitumour activity [35]. This activity (i) increases as a function of the size of the oligomers, (ii) is generally higher for the more basic conformer of each oligomeric species, and (iii) often, particularly for the largest oligomers, is greater than that of BS-RNase [35]. Moreover, when assayed on non-malignant cells, the oligomers were completely devoid of cytotoxicity (J. Matousek, G. Gotte, P. Pouckova, J. Soucek, T. Slavik, F. Vottariello and M. Libonati, unpublished work). They also lack any embryotoxicity, but show an aspermatogenic action similar to that of BS-RNase [35] (see below).

Aspermatogenic and embryotoxic activities of RNase A oligomers

In addition to its antitumour activity, BS-RNase shows aspermatogenic activity, embryotoxic action and immunosuppressive activity, the last of which probably has the physiological role of suppressing the female immune response against components of bull semen [110]. RNase A oligomers were tested for their possible aspermatogenic activity, and were found to show a level strikingly similar to that of dimeric BS-RNase [35]. In contrast with BS-RNase, RNase A oligomers have no embryotoxic effects [35].

Discussion of the biological activities of the oligomers of RNase A

What are the main points concerning the biological actions of the oligomers of RNase A that deserve to be analysed? One is the way in which the various multimers can enter cells to produce their action. No clear evidence is available concerning this point, or about the route taken by the oligomers from the extracellelular matrix to the cytosol of malignant cells. It is assumed that they could enter by endocytosis after adsorption to the polyanionic cell surface, as suggested for BS-RNase [111–113], with this binding being highly favoured by the polycationic nature of the RNase A oligomers. Experimental evidence supporting this model is provided by a basic variant of the acidic, non-cytotoxic RNase Sa from *Streptomyces aureofaciens*. This variant, in which five lysine residues replace five acidic residues, indeed becomes cytotoxic [114]. In contrast, the removal by site-directed mutagenesis of a highly positively charged portion from the N-terminus of the ribotoxin α-sarcin greatly decreases the cytotoxic activity of this protein [115].

How can a ribonuclease be cytotoxic after entering a cell? It is quite logical to ascribe cytotoxicity to the ability of the RNase to degrade the various RNA species of the cell. This possible explanation is supported by the finding that, although a ribonuclease must not necessarily be oligomeric to exert its cytotoxic activity, it is absolutely necessary that it retains its ribonucleolytic activity, as shown, for instance, by the cytotoxic action of onconase [100,116,117]. Therefore the requirement of catalytic activity for cytotoxicity is a second important point concerning the biological actions of RNase A oligomers.

In principle, intracellular ribonuclease activity could also be displayed by monomeric RNase A. However, it is well known that many monomeric ribonucleases are blocked in the cells by their strong interaction with cRI (cytosolic ribonuclease inhibitor), a 50 kDa acidic protein which binds RNase A with a K_i of (4.4–5.9) × 10^{-14} M [118]. Therefore a third important requirement for the cytotoxic action of an RNase molecule is that it must be able to escape the interaction with cRI [111,118]. Undoubtedly, the dimeric, trimeric or tetrameric nature of the RNase A oligomers should prevent these molecules from interacting with cRI, which can indeed bind strongly to RNase A and monomeric BS-RNase, but not to native, dimeric BS-RNase [119]. In this connection, it is worth mentioning that RNase A conjugated with poly(ethylene glycol) acquires significant antitumour activity against human melanoma transplanted in athymic nude mice [120].

Since the stability of the quaternary structure of RNase A multimers has not been characterized *in vivo*, uncertainty exists regarding the duration of their cytotoxic actions. The stability of these oligomers depends on many factors. One is the size of the molecule: we have already reported that under defined experimental conditions dimers are more stable than trimers, and trimers are more stable than tetramers [20]. Moreover, a difference exists between the two conformational isomers of each oligomeric species [20,39]. The stability of the oligomers also depends on their concentration in solution (dilute concentrations are more stable than concentrated solutions [20]), and on the environment (buffer, type of ions, pH, temperature, etc.) [20,39]. In addition, the dissociation of tetramers and trimers might not lead to sudden and complete inactivation, since smaller oligomers are transiently produced [19,20]. Finally, stability data obtained in a test tube cannot be simply extrapolated to *in vitro* or *in vivo* assays. In other words, when the oligomers are added to cell cultures or injected into neoplastic tissues, the environmental conditions and possible interactions with other molecules could influence their dissociation in unknown ways. Therefore, a fourth point deserving attention is the stability of the RNase A oligomers.

One final important point can be highlighted, i.e. the absence of any detectable embryotoxicity of the RNase A oligomers, which differentiates them from BS-RNase. The lack of embryotoxicity is an important property of the RNase A multimers, which makes them better potential antitumour agents than other ribonucleases. However, this fact might be deprived of real practical importance, because it could be ascribed, at least partly, to their metastability. In other words, the RNA oligomers may not survive in the cell long enough to produce embryotoxic effects.

Possible relationship between the antitumour activity of RNase A multimers and their ability to degrade dsRNA

Perhaps the most interesting point to consider is the striking similarity between the patterns of dsRNA depolymerization and the rank order of the antitumour action displayed by the RNase A oligomers. The larger oligomers are more active against dsRNA than the smaller oligomers: tetramers are more active than trimers, and trimers than dimers. Moreover, within each pair of conformers, the more basic one is more active against dsRNAs than its less basic counterpart. Similarly, the larger oligomers display greater antitumour activity, both *in vitro* and *in vivo*, than the smaller ones: tetramers are more active as antitumour agents than trimers, and trimers are more active than dimers. Here again, within each pair of conformers, the more basic one is generally more active than the less basic conformer, the only exception occurring in an *in vivo* experiment where the two dimers reversed their ranking, with the C-dimer (more basic) showing a lower activity than the N-dimer (Figure 11) [35]. Once more, the 'basic' character of the RNase A oligomers reveals its importance; it is possibly at the root of three events: the entrance of the oligomers into the cells, their ability to degrade dsRNAs and poly(A), and their antitumour action.

The similarity between the pattern of dsRNA degradation and the pattern of the antitumour activity of oligomeric RNase A species may not be a coincidence. In this regard, it is worth mentioning that dimers and higher oligomers of RNase A [121], as well as BS-RNase [122], were also found to be able to degrade DNA:RNA hybrids in addition to dsRNA. While it is unlikely that RNase molecules, especially RNase A oligomers, could enter the nucleus of a normal cell, neoplastic cells can be abnormal in their ability to select and regulate proteins for transport through the membrane. Moreover, the nuclear membrane breaks down during cell division, and cell division is definitely more frequent in cancer cells than in normal cells. In conclusion, both double-helical regions of cellular tRNA, mRNA or rRNA, and the hybrid DNA:RNA sequences, like those forming in the priming of DNA synthesis by RNA or during the synthesis of mRNA, may be good targets for the RNase A oligomers in their essentially uncontrolled action inside the malignant cells. The same may be true of the 3′ poly(A) tails of mRNA, which appear to be necessary for a normal half-life of mRNA, possibly by increasing its resistance to nuclease attack.

The ability of RNase A multimers to degrade double-helical regions of RNA molecules, DNA:RNA hybrid sequences and the 3′ poly(A) tails of mRNA, as well as their capacity to escape the interaction with cRI after entering a malignant cell, could be the reasons for their remarkable antitumour action.

CONCLUSIONS

The oligomerization of RNase A has proven to be a rather interesting phenomenon, from the point of view of both protein structure and the functional properties of the oligomers. Both aspects are still open to investigation. The structural versatility of RNase A is notable, and offers the potential to study other possible molecular products of its aggregation, and to improve our understanding of the variables controlling 3D domain swapping, which is the mechanism leading to the multiform oligomerization of the protein. Possible future research directions concern the solution of the structure of the NC-trimer and the identification of the small peaks identified as X, Y and Z in Figure 2(B). Moreover, the identification (notwithstanding their very modest amounts) of the larger oligomers (P and H in Figure 2B) could also be of some interest. On the other hand, the biological actions acquired by RNase A in its oligomeric form need to be studied in depth in order to clarify further the antitumour activity of the multimers and, in particular, their metastability *in vitro* and possibly *in vivo*. The survival of RNase A oligomers for a reasonable time is indeed the basis for any possible practical use of them as anticancer agents.

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