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Visions & Reflections

Biological actions of the oligomers of ribonuclease A

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Oligomeric RNase A

Bovine pancreatic ribonuclease A (RNase A, E.C. 3.1.27.5), one of the most extensively studied proteins [1–4], is a rather small molecule (124 amino acids; mass, 13,686 Da [4]), remarkably stable, and structurally and functionally very versatile [5, 6]. Its pI is 9.3 because of the prevalence of basic (10 Lys, 4 Arg, 4 His) over acidic (5 Asp, 5 Glu) residues, and its main physiological function is the digestion of foreign RNA [7]. Crestfield, Stein and Moore reported in their classic paper published 42 years ago [8] that RNase A can oligomerise when lyophilised from 50% acetic acid solution. These authors [8], and Fruchter and Crestfield [9, 10], characterised two dimeric conformers, envisaging the dimerisation of RNase A as due to the exchange of the N-termini between two RNase A monomers in such a way as to rearrange the correct geometry of the active site in the resulting dimer. Indeed, Crestfield et al. demonstrated that the two dimers have the same specific activity as native, monomeric RNase A on 2′,3′-cyclic cytidylate as substrate [8, 9]. The mechanism envisaged by Crestfield et al. [8–10], later generalised and called by Eisenberg et al. '3D [three dimensional] domain swapping' [11], is valid for one of the two known dimeric RNase A conformers, the N-dimer, formed by the swapping of residues 1–15 of each subunit [12]. The other conformer, the C-dimer, more basic than the N-dimer, was found to form by the swapping of the C-termini (residues 116-124) of two monomers [13]. RNase A, by swapping its N-terminus and/or C-terminus, also forms higher-order, linear or cyclic multimers, each species comprising at least two conformers, one less basic, one more basic [5, 6, 14–18]. Two trimers, the linear NC-trimer, less basic, and the cyclic, propeller-like C-trimer, more basic [17], as well as two linear tetramers, the less basic NCN-tetramer, and the more basic CNC-tetramer [16, 18, 19], have been characterised. Recently, two additional tetrameric conformers were identified and studied [20], whereas RNase A pentamers have also been described [5] but not yet characterised.

A comment is appropriate here. Protein aggregation is usually an abnormal, hurtful event for the protein as well as for the cells and tissues in which the protein aggregates: the structure and physical-chemical properties of the protein change, leading to its irreversible precipitation, with consequent damage to the affected tissue(s). Many severe neurodegenerative diseases, such as Alzheimer's, Parkinson's, prion diseases and so on, as well as the so-called polyglutamine extension diseases [21, 22], are characterised by the intra- and extracellular presence of large amounts of insoluble protein aggregates. However, it might be worth mentioning that the mature fibrils or amyloid plaques appear to be definitely less toxic than the intermediate pre-fibrillar aggregates [23]. On the contrary, the aggregation of ribonuclease A by 3D domain swapping does not damage the protein. The RNase A multimers show unchanged solubility properties in comparison with the monomeric enzyme, and a specific activity on cytidine 2′,3′-cyclic phosphate identical to that of native RNase A [8, 9]. Moreover, they acquire novel biological activities and the ability to degrade secondary structures of RNA [5, 6, 14].

Biological actions of the RNase A oligomers

Native, monomeric RNase A is devoid of any significant biological activity, although about 50 years ago Ledoux

reported that the enzyme (administered at high concentration) was cytotoxic on Ehrlich's and Krebs's carcinomas in mice, and Walker's carcinoma in rats [24, 25]. Instead, RNase A oligomers acquire a remarkable anti-tumour activity and an aspermatogenic action [19]. This may actually not be surprising if we take into account that cross-linked dimers [26, 27] and trimers [28] of RNase A, as well as monomeric RNase A conjugated with poly(ethylene glycol) [29], have been shown to be cytotoxic against several types of tumours. Moreover, the biological actions of dimers and higher oligomers of RNase A may become reasonable considering the strong anti-tumour and aspermatogenic activities displayed by bovine seminal ribonuclease (BS-RNase), which shares 83% identity with the pancreatic enzyme and is the only naturally dimeric RNase known thus far [30, 31].

Anti-tumour activity of oligomeric RNase A

The anti-tumour action of dimers, trimers and tetramers of RNase A has been tested in vitro and in vivo [19]. In vitro it was assayed on the proliferation of two human tumour cell lines, ML-2 and HL-60, derived from patients suffering from acute myeloid leukemia. The action of the RNase A multimers increases with the increase of their mass, and the two tetramers show generally higher activity values than those observed with BS-RNase. Moreover, within each pair of conformers, the cytotoxic action is stronger for the more basic conformer [19]. Recently, a comparative investigation of the effects that BS-RNase and onconase have on the proliferation of human ML-2 cells has been published [32]. Onconase is a powerful cytotoxic ribonuclease from the oocytes and early embryos of *Rana pipiens*. It is monomeric in nature and has about 30% identity to RNase A [31, 33–36]. The work by Matousek et al. [32], performed with the same methods and within the same range of enzyme concentrations used in the experiments carried out with the RNase A multimers [19], shows that onconase is more active than BS-RNase in inhibiting the proliferation of ML-2 cells. Interestingly, by comparing the available data [19, 32], the extent of inhibition due to the RNase A CNC-tetramer, the more basic one, appears to be even higher than that displayed by onconase. In vivo the anti-tumour activity of the RNase A multimers was tested on the growth of a human non-pigmented melanoma transplanted in nude mice. The inhibition of tumour growth increased, again, with the mass of the oligomers tested (which is consistent with the results of the in vitro experiments), was generally higher for the more basic conformer of each oligomeric species, and almost total as a result of the action of the two tetramers [19].

Aspermatogenic activity of oligomeric RNase A

The RNase A oligomers, from dimers to tetramers, are aspermatogenic [19], like BS-RNase and onconase [31]. Their action was tested, in comparison with that of BS-RNase, by injecting proper amounts of the RNase species in the left testis of mice [19]. The extent of aspermatogenesis induced by the RNase A multimers was similar to that shown by seminal RNase for two out of three parameters investigated, i.e., the width of the spermatogenic layers and the diameter of the seminiferous tubules of testes, whereas the index weight of mice testes was not affected. In the comparative investigation carried out by Matousek et al. [32], the aspermatogenic activity of onconase was definitely higher than that of BS-RNase. Therefore, onconase appears also to be markedly more aspermatogenic than the RNase A oligomers.

An interesting point: the RNase A multimers are not embryotoxic

An important point has to be highlighted, i.e., the absence of embryotoxicity in the action of the RNase A oligomers [19], in contrast with the embryotoxic effects ascertained for BS-RNase, and, in particular, for onconase [32]. This is quite interesting also in view of a possible future use of RNase A multimers as therapeutic agents. However, the absence of embryotoxicity in the action of oligomeric RNase A deserves to be discussed and carefully evaluated. The structural stability of dimers, trimers and tetramers decreases with the increase of the size of the oligomers [16], and can be highly influenced by changes in temperature, protein concentration, pH and ion composition of the medium [16, 37]. Moreover, at the moment nobody knows how the oligomers survive in cell culture or when injected into a tissue: their stability can also be affected by the interaction of the RNase A multimers with other molecules or generally with other components of the cell. In conclusion, the results of the embryotoxicity experiments have to be taken 'cum grano salis': we cannot exclude that the lack of embryotoxicity could be ascribed to the relative instability of the oligomers, i.e., to their possible short survival under the experimental conditions used. However, it has also to be taken into account that the dissociation of the higher, more active oligomers would not lead to immediate loss of activity, because transient oligomeric species (trimers, dimers) form [16, 20], that can exert similar, although weaker, biological actions [19].

Entrance of the oligomers of RNase A in the cells

How do RNase A multimers enter a cell? No experimental data are available; we can prognose only an analogic

proposal on the basis of the high similarity of bovine pancreatic RNase A with bovine seminal RNase. The latter enzyme seems to adsorb to the polyanionic glycosaminoglycans that cover the surface of mammalian malignant cells, entering the cell by endocytosis [38]. According to Bracale et al. [39], and Mastronicola et al. [40], from the extracellular matrix, where it concentrates, the enzyme would cross the cell membrane reaching, through the endosomes, the trans-Golgi network, and then, probably by crossing its membrane, the cytosol and/or the nucleoli. The RNase A multimers could follow a similar route. For such a mechanism, the basic charges on the surface of the entering protein are important [38]; and the oligomers of RNase A, as well as BS-RNase, are indeed very basic proteins. With regard to the importance of protein basicity, it is worth mentioning the work of Futami et al. [41] and of Ilinskaya et al. [42]. Futami et al. converted non-cytotoxic RNase A and human RNase 1 to very efficient cytotoxins by cationisation, and part of this effect was certainly due to improved internalisation of the two proteins [41]. Ilinskaya et al. found that a mutant of *Streptomyces aureofaciens* ribonuclease (RNase Sa), whose net charge was changed from –7 to +3 by substituting five acidic residues of the protein with five basic amino acids [43], is cytotoxic [42], possibly because, once again, of an increased interaction with the polyanionic surface of malignant cells and therefore a facilitated entrance.

Targets of the RNase A multimers in malignant cells

To be cytotoxic, a ribonuclease must keep its ribonucleolytic activity [44, 45]. Oligomers of RNase A, in comparison with the monomeric enzyme, show an unchanged activity on small substrates (cytidine 2′,3′-cyclic phosphate) [8,9], and a slightly reduced activity on single-stranded polyribonucleotides [5, 6, 14]. For the latter property a tentative interpretation has been advanced [5, 6]. The oligomers acquire instead a remarkable degrading action against double-stranded RNA (dsRNA) [5, 6, 14], DNA:RNA hybrids [6, 46], and the neutral or acidic forms of polyadenylate [poly(A)] [5, 6, 47, 48] that is lacking in monomeric RNase A. This activity also increases with the increase of the mass of the multimers, i.e., RNase A pentamers are more active than tetramers, tetramers than trimers, and trimers than dimers [5, 6, 14, 47]. Moreover, within each pair of conformational isomers, the more basic conformer is more active than the less basic one. A hypothesis, repeatedly confirmed [5, 6, 49, 50], was advanced years ago [49–51] to explain the action that ribonucleases more basic than RNase A show against dsRNA. It can also apply to the RNase A multimers. The hypothesis proposes that the positive charges on the surface of a ribonuclease, but particularly the basic charges specifically located at the active site region of the enzyme, induce the destabilisation of the secondary structure of RNA, such as to make, on interaction with the enzyme, a double-helix single-stranded and therefore susceptible to immediate degradation. Based on this view, the so-called single-stranded RNA species of the cell, i.e., ribosomal RNA (rRNA), messenger RNA (mRNA) and transfer RNA (tRNA), which actually bear a substantial amount of double-stranded structure, could become selectively sensitive to the basic RNase A oligomers, the extent of their action depending on the number and/or the density of positive charges present on the oligomers. But hybrid DNA:RNA sequences also play a physiological role in the cell, particularly in fast dividing, malignant cells. Indeed, they form at the first step of DNA replication as well as in the biosynthesis of mRNA. Moreover, eucaryotic mRNA molecules bear poly(A) tails linked at their 3′-ends, whose function was hypothesised to be a defence against the action of nucleases, therefore enhancing the half-life of mRNA. The efficient degrading action of the various RNase A multimers on dsRNA, DNA:RNA hybrids and poly(A) could therefore justify their efficient anti-tumour activity. In this connection, the ability to attack dsRNA, DNA:RNA hybrids and polyadenylate is also common to bovine seminal RNase [52, 53], whose strong cytotoxic action could also be due to these activities, in addition to the selective action on rRNA shown by the enzyme [31, 54].

A common root for the functional properties of the RNase A multimers?

The biological activities of oligomeric RNase A, as well as its ability to degrade dsRNA, RNA:DNA hybrids and poly(A), share the property to increase significantly with the increase of the mass of the oligomers. A common root of both these activities can therefore be the increasing number and/or density of positive charges of the RNase A multimers in going from dimers to higher oligomers. Basic charges appear to be important (i) for the entrance of the multimers in the cells, (ii) for the interaction of the internalised multimers with their target polyanionic molecules and (iii) for the action of the oligomers on the various cellular RNA species, in particular on their secondary structures; i.e., after all, for the cytotoxic activity of oligomeric RNase A toward malignant cells.

Why are RNase A oligomers cytotoxic, while monomeric RNase A is not?

A simple reason why RNase A oligomers, in contrast with monomeric RNase A, display a cytotoxic and an aspermatogenic activity could be their ability to escape interaction with the well-known cytosolic RNase inhibitor

(RI) [55] because of their oligomeric structure. Support to this explanation is given by the observation that dimeric BS-RNase loses its biological activities when it is artificially monomerised [56]. Dimerisation or generally oligomerisation appear to be the way by which a ribonuclease escapes interaction with RI and therefore becomes biologically active. However, this is true for dimeric or oligomeric bovine RNase A and for BS-RNase, which are mammalian ribonucleases, but neither for onconase, nor for the monomeric ribonuclease of *Rana catesbeiana* [44]. In these cases, the lack of significant inhibitory action by the mammalian RIs could actually be ascribed not to lack of interaction with the RNases, but to their very low affinity for ribonucleases from different species [44]. However, the idea that the biological actions of oligomeric ribonucleases might only be due to their ability to escape interaction with RI could be a too naive explanation. There might be other, not yet discovered, reasons that could be also responsible, at least in part, for the biological actions of these ribonucleases.

Conclusions and perspectives

Although (i) Park and Raines reported that dimers of RNase A may spontaneously form under physiological conditions (pH 6.5, and 37 or 65 °C), suggesting that they could exist in vivo [57]; and (ii) dimers and higher-order oligomers can also be obtained under definitely milder conditions [15] than those outlined by Crestfield et al. [8–10], the RNase A multimers as described and discussed here are artificial, not physiological molecules, and their biological actions must be considered to be side effects ensuing from the quaternary structure acquired by the protein molecule. RNase A dimers, trimers and tetramers show a remarkable in vitro and in vivo anti-tumour activity, whose extent is a function of the mass of the oligomers [19]. Dimers, trimers, tetramers and pentamers degrade single-stranded RNA [5, 14, 52]; moreover, they also degrade dsRNA, DNA:RNA hybrids and poly(A) with an efficiency that increases with the increase of the mass of the oligomers [46–52]. These activities may be responsible for the selective cytotoxic action of the oligomers toward tumour cells. The anti-tumour action and degrading activity on secondary structures of RNA and poly(A) can be impaired by the instability of the oligomers, which increases with the increase of their size. Both the biological and catalytic properties of the multimers can be correlated with the number and/or the density of positive charges present on the protein(s). Basic charges could also be determinant for the entrance of the RNase A oligomers in malignant cells, and their binding to the various cellular RNA species. However, experimental evidence concerning the entry and the route followed by the RNase A oligomers in the cell is lacking. Although an aspermatogenic action of the oligomers has been described in addition to their antitumour activity [19], much work has to be done to find whether the RNase A multimers are endowed with other properties, such as the immunosuppressive and immunogenic activities shown by bovine seminal RNase [31, 32]. The lack of embryotoxicity in the action of the RNase A oligomers also deserves to be carefully studied. Is it a real property of the RNase A multimers or the result of their metastable nature? Finally, the biological actions of oligomeric RNase A, in particular the strong anti-tumour activity shown by the higher oligomers, are also interesting from the point of view of the possible future use of the RNase A multimers as therapeutic agents. However, this possibility appears to be modest unless the problem of the uncertain survival of the oligomers in living cells and tissues can be solved. Their structural instability could be obviated by cross-linking [26–28], but this procedure would reduce the positive charges of the oligomers, and therefore, on the basis of the foregoing discussion, the efficiency of their action.

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