Second generation antitumour human RNase: significance of its structural and functional features for the mechanism of antitumour action

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A second generation mutant of dimeric human pancreas RNase (HHP2-RNase), was obtained by a single residue mutation (Glu¹¹¹ → Gly) of the previously described dimeric human pancreas RNase variant (HHP-RNase). HHP2-RNase was found to be a highly specific antitumour agent, with an enhanced cytotoxic activity compared with HHP-RNase. The structural and functional requisites of the antitumour action of HHP2-RNase were investigated and compared with those of other dimeric antitumour RNases. The stability of the dimeric structure, i.e. the resistance of human dimeric RNase variants to reductive cleavage of the two intersubunit disulphide bonds that bridge the subunits,

was determined to be an essential feature of antitumour dimeric RNases. The stability of the dimeric structure is in turn responsible for the resistance to inhibition by the cytosolic RNase inhibitor (cRI). Both the stability of the dimeric structure and the resistance to cRI inhibition appeared to be highly enhanced by an RNase substrate. This suggests a possible role for RNA in the amplification of the antitumour potential of dimeric RNases.

Key words: antitumour agents, protein engineering, ribonucleases, RNase inhibitor.

INTRODUCTION

Homologue-scanning mutagenesis is a powerful method for identifying sequences that cause functional variations among homologous proteins [1]. Following this strategy, monomeric RNases, only active as enzymes (i.e. devoid of any biological activities), such as bovine pancreas RNase (RNase A) and human pancreas RNase (HP-RNase) have been engineered into dimeric variants endowed with cytotoxic activity [2–5].

For these protein engineering experiments, bovine seminal RNase (BS-RNase) [6], the only dimeric RNase in the vertebrate superfamily, was used as a model protein. Its two identical subunits are linked by two disulphide bonds, and may, or may not, reciprocally exchange their N-terminal α -helical segments, thus generating two quaternary structures in equilibrium with one another: the exchanging M×M form, accounting for approx. 70% of the protein molecules, and the non-exchanging M=M form [7].

An unusual multiplicity of biological actions have been related to these structural peculiarities, as BS-RNase, whose physiological role remains unclear, is an antispermatogenic, antiviral, embryotoxic, immunosuppressive and antitumour agent [6,8]. The strong antitumour action of this protein, consisting of a selective toxicity towards malignant cells, has been well established *in vitro* and *in vivo* [9–13].

An investigation into the mechanism of BS-RNase antitumour action [11] has indicated that the protein enters the cells via an absorptive rather than a receptor-mediated internalization mechanism. Once internalized, BS-RNase apparently has access to different pathways in tumour and normal cells, as the protein only reaches the cytosolic compartment in tumour cells, and extensively degrades rRNA, which leads to cell death [11]. The structural features described above, i.e. the dimeric structure and the interchange of the N-terminal segments, have been reported to play key roles in the mechanism of antitumour action of BS-RNase [10,14,15].

The identification of the amino acid residues essential for the cytotoxic activity of BS-RNase was achieved using the homologue-scanning mutagenesis technique [1]. The substitution of a few amino acid residues of RNase A, a monomeric and non-cytotoxic RNase homologous to BS-RNase, with the corresponding residues of BS-RNase led to the production of covalent dimeric variants of RNase A [2,3], which were found to be specifically cytotoxic for malignant cells.

To obtain a human dimeric RNase, four residues of HP-RNase were substituted with the corresponding residues of BS-RNase (Gln²⁸ → Leu, Arg³¹ → Cys, Arg³² → Cys and Asn³⁴ → Lys) [4]. By these substitutions, helix-II of HP-RNase was engineered to reproduce helix-II of BS-RNase, which generates, with the corresponding segment of the partner subunit, the intersubunit interface of BS-RNase dimers [16]. The dimeric HP-RNase variant (HHP-RNase) was found to be selectively cytotoxic towards malignant cells [4].

In the present study, a new version of human dimeric RNase was prepared, based on the findings that the presence of glycine residues at positions 38 and 111 greatly enhanced the cytotoxic activity of the dimeric mutant of RNase A [17]. As a glycine residue is already present at position 38 in native HP-RNase, the substitution Glu¹¹¹ → Gly in the cDNA coding for the subunit chain of HHP-RNase was performed. This led to a second generation mutant of dimeric HP-RNase (HHP2-RNase), which is hyperactive as a cytotoxic agent and has been instrumental in a comparative investigation of the structural and functional determinants of antitumour RNases.

EXPERIMENTAL

General procedures

BS-RNase was purified from seminal vesicles as previously described [18,19]. Bacterial cultures, plasmid purification and

Abbreviations used: BS-RNase, bovine seminal RNase; cRI, cytosolic RNase inhibitor; DTT, dithiothreitol; DVS, divinyl sulphone; HP-RNase, human pancreas RNase; HHP-RNase, dimeric HP-RNase variant; HHP2-RNase, second generation mutant of dimeric HP-RNase; HP2-RNase, monomeric derivative of HHP2-RNase; RNase A, bovine pancreas RNase; SV40, simian virus 40; UpA, uridylyl(3',5')adenosine.

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bacterial transformation were performed as described previously [20]. Plasmid pET22b+ and *Escherichia coli* strain BL21DE3 were obtained from AMS Biotechnology (Milan, Italy). Double-stranded DNA was sequenced by the dideoxy method of Sanger et al. [21], using the Sequenase Sequencing Kit and labelled nucleotides from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). SDS/PAGE was carried out according to the method of Laemmli [22].

Construction and expression of HHP2-RNase

The cDNA coding for HP-RNase (Gln²⁸ → Leu, Arg³¹ → Cys, Arg³² → Cys and Asn³⁴ → Lys) [4], cloned into the *NdeI/HindIII* restriction sites of the expression vector pET22b+, was mutated as previously described [23] in order to replace the wild-type glutamic acid with a glycine codon. The mutagenic oligonucleotide 5'-CAGGTCGACTTCAAGTAGAGTCTTCAAC-AG-3' was synthesized by the Stazione Zoologica A. Dohrn (Naples, Italy). The mutation was verified by DNA sequencing.

The recombinant expression vector, containing the mutated cDNA, was used to transform BL21DE3 *E. coli* cells. The recombinant protein was expressed, isolated and dimerized as described previously [4]. Yields of HPLC-purified HHP2-RNase were approx. 5 mg/litre of the bacterial culture.

Cytotoxicity assays

Murine fibroblasts (Balb/c 3T3 cell line) and their counterpart obtained by transformation with simian virus 40 (SV40; SVT2 cell line) were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were seeded in 96 well plates (3 × 10³ cells/well in 100 µl of medium) and the assays were performed as described previously [11]. In brief, dose-response curves were obtained by measuring in triplicate the percentage of cell survival in the presence of the protein under study with respect to control cultures grown in the absence of the protein. Cell survival was determined either by counting the number of cells excluding Trypan Blue (Sigma, St. Louis, MO, U.S.A.), or by measuring the extent of protein biosynthesis using [³H]leucine (Amersham Pharmacia Biotech) incorporation.

Enzymic assays

Enzyme activity was tested by spectrophotometric assays, using a Cary 1E (Varian, Palo Alto, CA, U.S.A.) spectrophotometer. Activity with yeast RNA as the substrate was measured following the procedure described by Kunitz [24]. Activity with uridylyl(3',5')adenosine (UpA; Sigma) as the substrate, used at a concentration of 75 µM, was tested at 25 °C in 0.1 M Tris/HCl (pH 7.3) containing 0.3 M NaCl. Following the addition of the enzyme under test, the decrease in absorbance was recorded for 4 min at 286 nm. The concentrations of the enzyme were: 2.9 nM for BS-RNase and RNase A (subunit concentration), and 5.9 nM for HHP-RNase and HHP2-RNase (subunit concentration). Activity with poly(A)·poly(U) (100 µM, Sigma) as the substrate was tested in 100 mM MOPS buffer (pH 7.5) containing 0.1 M NaCl. The increase in absorbance was recorded for 0 to 1 min at 260 nm.

Determination of the isoform content

The strategy followed to isolate BS-RNase isoforms was that described previously [7], with minor modifications. The intersubunit disulphide bonds of HHP2-RNase were selectively reduced in 0.1 M Tris/acetate (pH 8.4) containing 0.3 M NaCl, with a 10-fold molar excess of dithiothreitol (DTT). After 20 min

at 25 °C, to verify that a complete reduction of the intersubunit disulphide bonds occurred, an aliquot of HHP2-RNase (5 µg) was alkylated with a 2-fold molar excess of iodoacetamide over DTT, kept for 5 min in the dark and analysed by SDS/PAGE. The protein was then analysed on an FPLC apparatus (Pharmacia Biotech, Uppsala, Sweden) by gel-filtration on a Superdex 75 Hi-load 10/30 column equilibrated in 50 mM ammonium acetate (pH 5) containing 0.3 M NaCl, at a flow rate of 0.3 ml/min. The areas of the eluted peaks were determined by the use of a chart recorder (C-R6A Chromatopac; Shimadzu, Kyoto, Japan).

Alternatively, the dimeric RNases (10 µM, subunit concentration) were reacted with divinyl sulphone [DVS; Sigma; 1 µl of a 10% (v/v) solution in diethyl ether] in a final volume of 100 µl of 0.1 M sodium acetate buffer (pH 5) containing 0.2 M NaCl. Following incubation for 72 h at 30 °C, the reaction was quenched by the addition of 2-mercaptoethanol at a final concentration of 0.2 M. The proteins were analysed by SDS/PAGE under reducing conditions. The relative amounts of monomeric and dimeric species were measured by a GS-710 Imaging Densitometer (Bio-Rad, Hercules, CA, U.S.A.), using Quantity One software.

Inhibition assays

The cytosolic RNase inhibitor (cRI), purchased from Takara Shuzo Co. Ltd (Kyoto, Japan), was diluted 5-fold with 0.1 M Tris/HCl (pH 7.4) containing 9 mM GSH, 0.15 mM GSSG and 10% (v/v) glycerol, and extensively dialysed at 4 °C against the same buffer. For inhibition assays, appropriate amounts of freshly dialysed cRI were added to 0.4 ml of 150 µM UpA in 0.1 M Tris/HCl (pH 7.3) containing 0.3 M NaCl, followed by the addition of the enzyme under test. Enzymic activity was measured as described above. The extent of inhibition was calculated with respect to the enzyme activity registered in the absence of cRI. As a control in all of the experiments performed, the inhibitory activity of dialysed cRI was tested in parallel on RNase A. RNase A was found to be fully inhibited under the conditions employed, by stoichiometric amounts of cRI.

Stability of the dimeric structure under reducing conditions

An available protocol [12] was employed, with minor modifications, as follows. HHP2-RNase (14 µM, subunit concentration) was incubated at 25 °C in 0.1 M Tris/acetate (pH 7.4) containing 9 mM GSH and 0.15 mM GSSG, in the presence or absence of 5.9 mM UpA. At appropriate time intervals, aliquots of 3 µg of protein were withdrawn, reacted for 5 min in the dark with a 3.7 molar excess of iodoacetamide over GSH, and then analysed by SDS/PAGE under non-reducing conditions. The relative amounts of monomeric and dimeric species were measured by densitometric analyses with an Enhanced Laser Densitometer (Ultrosan XL; LKB, Bromma, Sweden).

RESULTS

A comparison of the coding sequences of BS-RNase and engineered HHP-RNase [4] suggested, on the basis of the data reported in the literature [17], that we should further engineer HHP-RNase by substituting the glutamic acid at position 111 with a glycine residue, i.e. the residue present at that position in BS-RNase subunit chain. The mutated cDNA was expressed, and the recombinant protein was isolated and dimerized as previously reported for HHP-RNase [4]. The recombinant protein, harbouring five mutations with respect to the parent HP-RNase, i.e. Gln²⁸ → Leu, Arg³¹ → Cys, Arg³² → Cys, Asn³⁴ →

Figure 1 HHP2-RNase is selectively cytotoxic for malignant cells

The survival of SVT2 malignant fibroblasts (continuous lines) was tested on cells grown for 48 h in the presence of HHP2-RNase (■), HHP-RNase (□), BS-RNase (▲) or HP2-RNase (○). The survival of Balb/c 3T3 normal fibroblasts (broken lines), grown in the presence of HHP2-RNase (■) or BS-RNase (▲) is also shown. Results are presented as the means $\pm$ S.D. from three experiments.

Table 1 Enzymic and cytotoxic activities of human dimeric RNases

The enzymic activity values are expressed for each substrate as the percentage of the specific activity calculated for native HP-RNase. These were: 51.1 ± 8.4 Kunitz units/mg for yeast RNA [24]; 244.0 ± 6.7 units/mg for UpA (where 1 unit = change in A_{286}/min per total measurable change) and 405.0 ± 4.5 units/mg for poly(A):poly(U) (where 1 unit = change in A_{260}/min per total measurable change). Cytotoxic activities are expressed as IC_{50} values, i.e. the protein concentrations inducing 50% cell death.

Protein	Enzymic activity			Cytotoxic activity IC_{50} ($\mu\text{g}/\text{ml}$)
	Yeast RNA	UpA	Poly(A):poly(U)	
HP-RNase	100	100	100	> 200
HHP-RNase	36 ± 3.1	29 ± 4.6	10 ± 2.1	30 ± 3.3
HHP2-RNase	46 ± 1.2	36 ± 1.6	9 ± 1.7	16 ± 0.6
BS-RNase	75 ± 2.4	96 ± 4.2	1 ± 0.3	25 ± 3.1

Lys and Glu¹¹¹ $\rightarrow$ Gly, was found to be a stable and enzymically active covalent dimer (see below). This protein was named HHP2-RNase.

When HHP2-RNase was tested on malignant SVT2 murine fibroblasts (Balb/c 3T3 cells transformed with SV40), it was found to be highly cytotoxic. In fact, the dose-response curves shown in Figure 1 for HHP-RNase, HHP2-RNase and BS-RNase, indicated that HHP2-RNase is the most active antitumour agent among dimeric RNases. The corresponding IC_{50} values, defined as the protein concentrations inducing 50% cell death, are tabulated in Table 1. Similar results were obtained from growth curves of SVT2 cells grown in the presence of the RNases, or by evaluating the cytotoxic effects of RNases in terms of inhibition of protein synthesis (results not shown).

These results indicate that the substitution Glu¹¹¹ $\rightarrow$ Gly into the HHP-RNase subunit chain enhances the RNase antitumour activity. Interestingly, as for the other antitumour RNases, the

Figure 2 Stability of the HHP2-RNase dimeric structure under reducing conditions

We tested the effects of a GSH/GSSG redox mixture on the dimeric structure of HHP2-RNase (■) and on BS-RNase isoforms M $\times$ M (○) and M=M (◇). The percentage of covalent dimeric species was determined by densitometric analysis of SDS/PAGE patterns obtained with the tested proteins (3 μg), run under non-reducing conditions. Proteins (14 μM , subunit concentration) were previously incubated for the indicated times in 0.1 M Tris/acetate (pH 7.4) containing 9 mM GSH and 0.15 mM GSSG, in the absence (A) or in the presence (B) of 5.9 mM UpA.

cytotoxic action of HHP2-RNase was also found to be highly selective for malignant cells, as the protein was thoroughly inactive toward the parental non-malignant cell line (i.e. 3T3 fibroblasts), tested in the same experiments (see Figure 1).

In an attempt to elucidate the key structural and functional features responsible for the antitumour action of human dimeric RNases, the requisites previously identified as essential (see the Introduction section) for the antitumour action of BS-RNase [8] were analysed.

Dimeric structure

A monomeric derivative of HHP2-RNase (HP2-RNase) was prepared, in which cysteine residues 31 and 32, responsible for the intersubunit disulphide bonds in dimeric RNases, were protected by two GSH moieties. When HP2-RNase was tested on malignant cells, it was found to be completely inactive (see Figure 1). This is in line with the results obtained with monomeric derivatives of natural and engineered RNases [4, 11], and indicates that the dimeric structure is an essential requisite for the antitumour action of HHP2-RNase.

We subsequently tested the ability of human dimeric RNase variants to survive as covalent dimers under conditions that mimic those present within the cytosolic compartment [25]. The proteins were incubated at 25 °C in 0.1 M Tris/acetate buffer (pH 7.4) in the presence of GSH and GSSG, at a molar ratio of 60:1. At different time intervals aliquots were withdrawn, iodoacetamide was added to alkylate the exposed thiols, and samples were analysed by SDS/PAGE under non-reducing conditions. To calculate the relative amounts of monomeric and dimeric species, densitometric analysis of the samples was performed.

We found that after 10 min of incubation approx. 75% of HHP2-RNase maintained a dimeric structure, and after 24 h more than 30% of the molecules were still dimeric (see Figure 2A). This indicates that a consistent fraction of human dimeric RNase molecules can survive as dimers, when exposed to a cytosolic-like reducing environment. Identical results were obtained with HHP-RNase (results not shown).

Figure 3 HHP2-RNase subunits have a low propensity to interchange their N-terminal segments

The content of exchanging dimers in HHP2-RNase and BS-RNase was analysed by gel-filtration (A and B) or by SDS/PAGE analyses (C). HHP2-RNase (A) or BS-RNase (B) (25 μ g), after the selective reduction of the intersubunit disulphide bonds with DTT, was analysed by FPLC gel-filtration on a Superdex 75 Hiload 10/30 column. D and M indicate the elution of non-covalent dimers and monomers respectively. (C) Analysis by SDS/PAGE under reducing conditions of RNases treated with DVS as a cross-linking reagent. Lane 1, molecular mass standards (BS-RNase A); lane 2, cross-linked HHP2-RNase; lane 3, cross-linked BS-RNase.

These results were compared with those obtained in parallel experiments with BS-RNase quaternary forms, $M \times M$ and $M = M$, which were freshly isolated. As shown in Figure 2(A), the quaternary isoform $M \times M$, in which the two subunits reciprocally exchange their N-terminal α -helices, is much more resistant to dissociation into subunits than the non-exchanging form, $M = M$. The different stabilities of BS-RNase isoforms to a mild redox mixture indicates, as previously reported by Raines and co-workers [12], that the propensity of BS-RNase isoforms to undergo monomerization, and hence the susceptibility of the intersubunit disulphide bonds to reduction, is strongly influenced by the quaternary conformation of the protein. It must be noted that under the same conditions HHP2-RNase and HHP-RNase (results not shown) were found to be more resistant to dissociation than the $M = M$ isoform, and less resistant than the $M \times M$ isoform.

When the experiment illustrated in Figure 2(A) was repeated in the presence of a typical RNase substrate, such as UpA (150 μ M), we found that both for HHP2-RNase and the BS-RNase isoforms the fraction of dimeric species was significantly

increased by the presence of the substrate (see Figure 2B). Identical results were also obtained with HHP-RNase (results not shown). These results indicate a protective effect of the substrate towards monomerization of the protein.

Interchange of the N-terminal domains

Given (1) the dependence of BS-RNase antitumour activity on the presence of the biologically active $M \times M$ quaternary isoform [14,15], and (2) the different stabilities of the $M \times M$ and $M = M$ isoforms to the reducing environment reported above, the presence of exchanging dimers in human dimeric RNases was analysed, following the protocol established for BS-RNase [7]. Briefly, the assay for discriminating between the exchanging ($M \times M$) and the non-exchanging ($M = M$) form is based on the different molecular size of their respective products as obtained by selective reduction of the intersubunit disulphide bonds. The product from the $M = M$ form is monomeric, as there is no exchange of the N-terminal α -helices, whereas it is dimeric (non-covalent dimers) from the $M \times M$ form, in which exchange occurs. The relative amounts of the two species, separated by gel-filtration, correspond to the contents of the $M \times M$ and $M = M$ isoforms in the protein.

In repeated experiments performed on HHP2-RNase, we found that only a small fraction ($\leq 15\%$) of the protein generated non-covalent dimers after the selective cleavage of the intersubunit disulphide bonds with DTT. The results of a typical experiment are shown in Figure 3(A), where more than 85% of the protein was eluted in the monomeric fraction. Identical results were obtained with HHP-RNase (results not shown). As a control, the results obtained with BS-RNase, a natural mixture of approx. 70% exchanging and 30% non-exchanging dimers, are shown in Figure 3(B).

An alternative assay was performed to confirm these data. This is based on the use of the cross-linking agent DVS, whose electrophilic CH_2 groups are able to cross-link the RNase active-site histidine residues (His¹² and His¹¹⁹), as demonstrated for BS-RNase [26]. In a dimer, in which the interchange of the N-terminal α -helices does not occur, the two histidine residues belong to the same polypeptide chain, whereas if there is interchange of the N-terminal ends, composite active sites are generated, and the histidine residues belong to different subunits. Thus upon reaction with DVS, the subunits of an exchanging dimer will be covalently linked to one another, even after reduction of the intersubunit disulphide bonds, whereas in a non-exchanging dimer the reduction will generate monomers.

Cross-linked HHP2-RNase was analysed by SDS/PAGE under reducing conditions (see Figure 3C). Two pairs of bands were detected, the upper corresponding to the dimeric fraction, i.e. to the N-terminal exchanging dimers, and the lower to the monomeric fraction. The smaller components present in both fractions were due to DVS-induced cleavages of the C-terminal end of the RNase [26]. The results of the experiment indicated that the percentage of the dimeric species, calculated by densitometric analysis, was approx. 12%, a value that confirms the low propensity of HHP2-RNase to interchange the N-terminal ends. For BS-RNase, analysed under identical conditions as a control, the dimeric species represented approx. 70% of the protein, the expected value for the fraction of BS-RNase $M \times M$ dimers.

No increase in the fractions of exchanging dimers of human dimeric RNases was detected (results not shown) when the proteins were extensively incubated either at 37 or 60 $^{\circ}\text{C}$ in the conditions described to favour the formation of the $M \times M$ dimers of BS-RNase [7,27]. Furthermore, again in contrast with

Figure 4 Effect of cRI on HHP2-RNase enzymic activity

(A) Inhibition curves of RNase A (2.9 nM; ●), HHP2-RNase (5.9 nM; ■) and BS-RNase (2.9 nM; ▲), obtained by increasing the ratio of cRI to RNase subunits. The enzymic activities were tested using UpA as a substrate at a final concentration of 0.15 mM. (B) Inhibition of HHP2-RNase (5.9 nM) activity by a 2-fold molar excess of cRI over the HHP2-RNase subunit concentration, tested in the presence of increasing concentrations of UpA (0.15–1.2 mM). In (A) and (B) the inhibition values were calculated with respect to the enzyme activity registered in the absence of cRI.

the results obtained for BS-RNase [28], an extensive incubation of HHP2-RNase with a large molar excess (400-fold) of the RNase substrate UpA did not favour the formation of exchanging dimers (results not shown).

These results indicate that human dimeric RNase variants have a much lower propensity to interchange their N-terminal segments than BS-RNase. Since human dimeric RNases are highly effective as cytotoxic agents, we conclude that the interchange of the N-terminal domains does not play any special role in determining their antitumour action.

Enzymic activity

Engineered HP-RNases were tested for catalytic activity on yeast RNA by the Kunitz assay [24]. The results are shown in Table 1. Both engineered RNases showed a reduced activity compared with that of the parent monomeric HP-RNase. This is in line with the results previously reported for native and recombinant dimeric RNases [17,29]. Similar results were obtained when human dimeric RNases were tested on UpA.

A more significant decrease in the enzymic activity compared with monomeric natural HP-RNase was found when the engineered RNases were tested on an analogue of double-stranded RNA, poly(A)·poly(U), a substrate efficiently degraded by native HP-RNase. Both human dimeric RNases were found to retain only approx. 10 % of the enzymic activity of the native monomeric protein, thus approaching the enzymic activity of BS-RNase (see Table 1).

These data indicate that the higher antitumour activity of a dimeric RNase cannot be ascribed to an enhanced efficiency in degrading single- or double-stranded RNA molecules.

Resistance to cRI

The cRI has a high affinity for monomeric pancreatic-type RNases [30,31]. Its cellular location provides the reducing environment that is essential to maintain its activity, based on the reduced state of its cysteine thiol groups. For this reason, *in vitro* tests on cRI inhibiting activity strictly require the presence

of a high concentration of a reducing agent, and DTT, a strong, non-physiological reducing agent, is routinely used. Such high concentrations of DTT could affect the stability of the inter-subunit disulphide bonds of the protein; furthermore, conditions mimicking those of the cytosol were considered preferable.

Thus we tested the sensitivity of human dimeric RNases to cRI inhibition under pseudo-physiological reducing conditions [25], i.e. in the presence of the GSH/GSSG mixture described above. The cRI preparation, provided by the manufacturers as a solution containing 5 mM DTT, was extensively dialysed against 0.1 M Tris/HCl (pH 7.4) containing 10 % (v/v) glycerol, 9 mM GSH and 0.15 mM GSSG. Freshly dialysed cRI was tested on HHP2-RNase, using 0.15 mM UpA as a substrate.

As shown in Figure 4(A), when HHP2-RNase activity was tested in the presence of increasing concentrations of cRI, it was found to be only partially susceptible to inhibition. Even in the presence of an excess of inhibitor, approx. 50 % of the original enzyme activity was still detectable. Identical results were obtained with HHP-RNase (results not shown). In contrast a monomeric RNase, such as RNase A (see Figure 4A), tested in the same experiment, was found to be fully inhibited in the presence of virtually stoichiometric amounts of cRI. Also in contrast, BS-RNase was found to be poorly inhibited (see Figure 4A), as previously reported [28].

The effect of RNA substrates on the inhibitory activity of cRI towards HHP2-RNase was then tested. In the presence of a 2-fold molar excess of cRI over the HHP2-RNase subunit, and of increasing concentrations of UpA as a substrate, we found that cRI inhibition markedly decreased as a function of UpA concentration (see Figure 4B), so that in the presence of 1.2 mM UpA more than 80 % of HHP2-RNase molecules retained their enzymic activity. Presumably, as reported for BS-RNase [28], the interaction of substrate molecule(s) with HHP2-RNase active site(s) and/or extra site(s) hinders the formation of the enzyme–cRI complex.

DISCUSSION

By protein engineering, the essential requisites responsible for the antitumour activity of BS-RNase were transferred to a human RNase scaffold, HP-RNase. This led to a recombinant human dimeric protein, HHP-RNase [4], capable of selectively killing tumour cells. In the present study we describe a second generation mutant, named HHP2-RNase, obtained by a single residue mutation in the HHP-RNase subunit chain (Glu¹¹¹ → Gly). This mutation enhanced the RNase cytotoxic activity, fully preserving the high specificity for malignant cells ascribed to the previous mutant. Possibly, the elimination of a negative charge (Glu¹¹¹) on the dimeric molecule favours the internalization and/or the transport of the protein to the cytosolic compartment of malignant cells. As for the selectivity of the cytotoxic action of human dimeric RNases, we may surmise, as already suggested for BS-RNase [8], that different intracellular pathways and/or interactions with extra- or intra-cellular targets allow the proteins to discriminate between malignant and normal cells.

An inspection of the structural and functional features potentially responsible for the antitumour action of human dimeric RNases clearly indicated that their cytotoxic activity cannot be ascribed to either the propensity of the dimer subunits to interchange structural elements, or the ability to efficiently degrade single- or double-stranded RNAs.

In fact, unlike BS-RNase, human dimeric RNases are found, by two independent experimental approaches, to have a low propensity to exchange the N-terminal segments between sub-

units, a feature that was described as an important requisite for the antitumour activity of BS-RNase [14].

On the other hand, human dimeric RNases are significantly less active on single- and double-stranded RNA substrates compared with their native monomeric counterpart, as it was also found for native and recombinant bovine RNases [17,29]. Possibly, the elimination of two positive charges (those of Arg³¹ and Arg³²), which in native HP-RNase contribute to the enzymic activity on double-stranded RNA [32], is responsible for the decreased activity of human RNase dimers on double-stranded RNA.

The results reported in the present study on human dimeric RNases established the importance of the dimeric structure in the antitumour action of HHP2-RNase, fully confirming the earlier proposed paradigm 'dimeric structure = antitumour action' [2,4,10,15].

However, the correlation between dimeric structure and antitumour action has to be included in the more general correlation linking the cytotoxic action of an RNase to its ability to escape cRI inhibition. To be cytotoxic an RNase must enter a cell and survive as an enzymically active protein, to effectively degrade cellular RNAs. Natural and engineered antitumour RNases show a common feature, i.e. the ability to escape cRI inhibition. The two natural antitumour RNases, BS-RNase and onconase, are poorly inhibited by cRI [8,12,28,33]. Monomeric RNases become cytotoxic agents when engineered into cRI-resistant RNases [5,34,35]. In the present study, dimerization of naturally monomeric HP-RNase transformed it into an anticancer RNase. When tested for the susceptibility in a pseudo-physiological reducing environment [25] to cRI inhibition, human dimeric RNases were found to be poorly inhibited.

We may argue that a dimeric RNase evades inhibition, since most of the contact sites with the inhibitor [36] are buried at the intersubunit interface of the protein; that is, a dimeric RNase does not fit in the horseshoe cavity of cRI. In this regard, the existence of a fraction of HHP2-RNase molecules susceptible to inhibition should be related to a partial monomerization under the conditions used in the assays. Thus the stability of the dimeric structure, which is maintained by two intersubunit disulphide bonds, is of crucial importance when exposed to mild reducing conditions similar to those present in the cytosol. We may presume that the more resistant an RNase dimer is in a reducing environment, the stronger its cytotoxic activity.

We tested the stability of the dimeric structure of human RNase variants to conditions that mimic those present in the cytosol. We found that human dimeric RNases are significantly stable, even after hours of incubation. When BS-RNase isoforms were tested as controls, we found different behaviours for M×M and M=M isoforms. M×M is much more stable as a covalent dimer than M=M when exposed to a mild reducing environment. These results, which confirm the data previously reported by Kim et al. [12], indicate that the exchange of the N-terminal segments in the M×M isoform greatly contributes to the resistance of the intersubunit disulphide bonds to reduction. This, in turn, has to be related to the stronger cytotoxic activity ascribed to the M×M form compared with the M=M form [14,15]. We may argue that the interchange of the N-terminal segments between the M×M subunits, which implies a crossing-over of the subunit chains, shields in some way the S-S bridges positioned at the intersubunit interface, and renders them less susceptible to reduction by GSH.

The stability of human dimeric RNases, for which no significant propensity to exchange the N-terminal domains was detected, is higher than that showed by the homologous non-exchanging conformer of BS-RNase, M=M. This suggests that

less exposed and/or less reactive intersubunit disulphide bonds may render human RNase mutants stable dimers that are poorly inhibited by cRI, and hence are active as cytotoxic agents.

Thus the dimeric structure of an antitumour RNase is an essential, but not sufficient, feature for its antitumour action. Rather, the stability of the dimeric covalent structure in the cytosolic compartment, and hence the ability to evade cRI, is the crucial requisite. BS-RNase acquires this requisite upon the interchange of domains; human dimeric RNases, instead, preferentially fold in a non-exchanging, but sufficiently stable, conformation so that, even after hours of incubation in a pseudo-physiological environment, a significant fraction of the molecules survives as dimers and evades cRI inhibition. However, to explain the partial inhibition of human dimeric RNases by cRI, we cannot rule out the possibility that this is due to the ability of cRI to bind only one protomer of human dimeric RNases, thus allowing the second protomer to be fully active as an enzyme.

The results reported in the present study also suggest that in the mechanism of action of native and recombinant dimeric RNases the RNA plays an important role. In the presence of UpA the two strictly related features for a cytotoxic RNase, i.e. the intracellular stability of the dimeric structure and the ability to evade cRI inhibition, are both greatly enhanced. This confirms the close correlation of these two key requisites, and suggests that RNA fragments may amplify the antitumour potential of cytotoxic RNases.

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