Purification and characterization of truncated ribonuclease inhibitor

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A recombinant pig ribonuclease inhibitor (Δ r-RI) lacking 90 or 93 *N*-terminal amino acid residues was isolated from a preparation of recombinant inhibitor. The kinetic parameters for the inhibition of ribonuclease A by Δ r-RI were determined and found to be only slightly altered in comparison with the full-length inhibitor. The deletion did, however, affect the surface properties of RI. The results are discussed in relation to those obtained by Lee & Vallee [(1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1879–1883].

INTRODUCTION

The primary structures of the ribonuclease (RNAase) inhibitor (RI) from human placenta (Lee *et al.*, 1988; Schneider *et al.*, 1988) and pig liver (Hofsteenge *et al.*, 1988) have been determined, and were found to be built from 15 homologous repeating units (leucine-rich repeats). Since only one molecule of RNAase is bound per molecule of RI, it is of interest to know which repeats are directly involved in the interactions between the two molecules. Lee & Vallee (1990*a,b*) have approached this problem by using deletion mutagenesis and have shown that considerable portions of the human RI molecule can be removed without destroying its inhibitory activity.

During the isolation of recombinant RI (r-RI), we were able to purify an *N*-terminally truncated form of the inhibitor (Δ r-RI). The inhibitory properties of this form of the molecule have been characterized. The results of these studies, together with those of Lee & Vallee (1990*b*), allow the binding site for RNAase on RI to be delineated.

EXPERIMENTAL

Materials

Bovine pancreatic RNAase A and uridylyl-3',5'-adenosine (UpA) were from Boehringer, Mannheim, Germany, and Sigma, St. Louis, MO, U.S.A. respectively, and were purified as described previously (Vicentini *et al.*, 1990). Tween 20 was from Bio-Rad Laboratories, Richmond, CA, U.S.A. All other chemicals were of the highest grade commercially available.

Production of recombinant RI

Saccharomyces cerevisiae, strain GRF 18 (α -his3-11 his3-15 leu2-3 leu2-112 can^R; Hinnen *et al.*, 1978) was transformed with the expression plasmid pJDB207/PHO5-RI. One of the transformants described previously (Vicentini *et al.*, 1990) was used to produce a 30 litre culture at 30 °C. Cells were collected by centrifugation, disrupted (Vicentini *et al.*, 1990), and stored at –20 °C. r-RI was purified by affinity chromatography on RNAase–Sephacrose, using 4 M-NaCl to elute the protein. Final purification was achieved by ion-exchange chromatography (Hofsteenge *et al.*, 1988).

Protein chemistry

All protein chemical procedures were performed as described (Hofsteenge *et al.*, 1988; Vicentini *et al.*, 1990).

Binding and inhibition assays

The binding of purified RI to immobilized RNAase was examined by the method of Lee & Vallee (1990*b*). To 2 μ g of RI in 50 μ l of Tris-buffered saline containing 1 mM-dithiothreitol and 0.1% poly(ethylene glycol) was added 10 μ l of RNAase–Sephacrose (2.4 mg of RNAase/ml of resin) in Eppendorf tubes, followed by incubation in an Eppendorf model 5432 shaker for 15 min. The beads were then collected by centrifugation, washed and treated with 2 mM-*p*-hydroxymercuribenzoate (pHMB) to elute RI. RI was detected by SDS/PAGE.

Kinetic studies of the inhibition of RNAase by RI were performed by the methods described before (Vicentini *et al.*, 1990), with the inclusion of 0.02% (w/v) Tween 20 in the reaction buffer to prevent adsorption of RI to the polyethylene tubes. The slow, tight-binding, inhibition of RNAase by RI can be adequately described by the following scheme (Lee *et al.*, 1989; Vicentini *et al.*, 1990):

where K_i is the inhibition constant and k_{on} and k_{off} are the association and dissociation rate constants respectively. Data obtained in the presence of five different concentrations of RI were fitted to eqn. 1 of Vicentini *et al.* (1990) by non-linear regression to yield estimates of K_i , k_{on} and k_{off} (Stone & Hofsteenge, 1986).

RESULTS

Recombinant pig RI expressed in yeast (r-RI) is virtually indistinguishable from RI isolated from pig liver (Vicentini *et al.*, 1990). The major difference between the two was the presence of proteolytically degraded forms in the recombinant preparation. Full-length r-RI could, however, readily be separated from these shortened forms by anion-exchange chromatography.

A large-scale (30 litre) preparation of r-RI was found to contain an unusually high amount (40%) of degraded forms (Fig. 1) in comparison with laboratory cultures (1 litre). The exact reason for this was unclear, but it could be the result of the higher temperature of fermentation (30 °C), and of increased handling times of the larger volumes. Analysis, by SDS/PAGE, of the material eluting from the Mono Q column (Fig. 1) showed that peak 1 contained truncated RI, whereas peak 2 contained full-length RI. The two species differed in apparent molecular mass by approx. 6000 Da. Moreover, gel filtration of Δ r-RI

Abbreviations used: RI, ribonuclease inhibitor; r-RI, recombinant RI; Δ r-RI, *N*-terminally truncated form of r-RI; UpA, uridylyl-3',5'-adenosine; pHMB, *p*-hydroxymercuribenzoate.

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Fig. 1. Anion-exchange chromatography of r-RI

Approx. 0.5 mg portions of r-RI, obtained by affinity chromatography as described in the Experimental section, were loaded on to a Mono Q column (HR5/5) and eluted with a gradient of NaCl. Fractions were pooled as indicated by the bars: peak 1, Δr-RI; peak 2; r-RI. The inset shows binding of RI to RNAase-Sephadex. In an analytical experiment, r-RI or Δr-RI (2 μg) was bound to RNAase-Sephadex. The beads were washed, and treated with 2 mM-pHMB to elute RI, as described in the Experimental section. The proteins in the eluate were loaded on to a 12.5%-polyacrylamide gel in the presence of SDS. Lane 1, Δr-RI; lane 2, r-RI.

Table 1. Amino acid composition of Δr-RI and its C-terminal peptide

The peptide containing residues 435–456 was obtained from Δr-RI by cleavage at Trp-434 and purified by reversed-phase h.p.l.c. (Vicentini *et al.*, 1990). Amino acid analysis was performed as described (Hofsteenge *et al.*, 1988). The numbers in parentheses are those expected from the sequence. N.D., not detected.

Amino acid	Δr-RI-(435–456)	Δr-RI
Asp	0.7 (1)	27.2 (30)
Glu	4.8 (5)	49.4 (46)
Cys		11.5 (24)
Ser	2.1 (2)	32.9 (33)
Thr	1.0 (1)	17.8 (18)
Gly	2.0 (2)	32.7 (33)
Ala	1.1 (1)	29.1 (28)
Arg	2.1 (2)	18.7 (18)
Pro	1.0 (1)	11.5 (13)
Val	1.7* (2)	14.5 (15)
Met		N.D. (1)
Ile	0.7* (1)	5.5 (6)
Leu	3.0 (3)	84.0 (80)
Lys	1.1 (1)	11.0 (12)
His		4.3 (4)
Tyr		2.9 (3)
Trp		N.D. (4)

* These values are low due to the presence of a Val-Ile bond.

indicated it to be monomeric. N-Terminal sequence analysis of reduced and carboxymethylated Δr-RI yielded two sequences. The major one (66%; N-Ser-Leu-Thr-Glu-Ala-Gly-Cys-) started at residue 91, whereas the minor one (34%; N-Glu-Ala-Gly-Cys-Gly-Val-Leu) was three amino acids shorter (starting at residue 94). The C-terminal portion of the molecule was characterized by cleavage at tryptophanyl residues, followed by peptide mapping using reversed-phase h.p.l.c. (Vicentini *et al.*, 1990). One C-terminal peptide was found, and amino acid analysis (Table 1) and sequencing showed it to encompass residues 435–456. No degraded forms of this peptide were found. Moreover, the amino

Fig. 2. Inhibition of RNAase A

(a) RNAase A (100 pM) was incubated with the indicated amount of r-RI. Residual RNAase activity was measured using UpA. Δr-RI (■) and r-RI (▲) were incubated in the absence of Tween 20; Δr-RI (□) was also incubated in the presence of 0.02% (w/v) Tween 20. (b) Progress curves of the hydrolysis of UpA (1 mM) by RNAase (10 pM) were determined as described in the Experimental section. The amount of r-RI was varied: ■, 0 pM; ▼, 4.8 pM; ▲, 9.6 pM; ▽, 14.4 pM; □, 19.2 pM. The lines show the best fit of the data to the equation describing slow, tight-binding, inhibition. For sake of clarity, the first four data points are not shown, but these points were included in the data analysis. U>p, uridine cyclic 2',3'-phosphate.

Table 2. Kinetic constants of the inhibition of RNAase A

The values of the kinetic constants for Δr-RI were obtained from the data in Fig. 2(b); those for r-RI are from Vicentini *et al.* (1990). The binding energy ($-\Delta G_b^0$) was calculated from the relationship $-\Delta G_b^0 = RT \cdot \ln K_i$, where R is the gas constant and T the absolute temperature.

	K_i (fM)	$10^{-8} \times k_{on}$ ($M^{-1} \cdot s^{-1}$)	$10^5 \times k_{off}$ (s^{-1})	$-\Delta G_b^0$ ($kJ \cdot mol^{-1}$)
r-RI	67 ± 4	1.5 ± 0.2	8.3 ± 0.2	75.2
Δr-RI	154 ± 14	1.80 ± 0.05	28 ± 3	73.2

acid composition of Δr-RI was consistent with the sequence data (Table 1). Therefore it can be concluded that Δr-RI is a mixture of two polypeptide chains, one missing 90 and the other missing 93 N-terminal amino acid residues. This part of the polypeptide chain comprises the N-terminal tail, repeats A₁ and B₁, and the majority of repeat A₂ (for nomenclature see Hofsteenge *et al.*, 1988, and Fig. 3).

Lee & Vallee (1990a) have made deletion mutants of human placental RI and found that removing the *N*-terminal residues yields a molecule that no longer binds to or inhibits RNAase A. The binding of Δ r-RI and r-RI to RNAase-Sephadex followed by elution with 2 mM-pHMB was examined in an analytical experiment. The results (Fig. 1 inset) show that purified r-RI (lane 2) and Δ r-RI (lane 1) can bind to RNAase-Sephadex, and that they can be eluted with pHMB. The binding was found to be specific, since inclusion of free RNAase (0.8 mg/ml) in the assay completely abolished binding to the immobilized enzyme (results not shown).

To investigate whether Δ r-RI was also able to inhibit RNAase, 100 μ M of enzyme was preincubated with increasing amounts of Δ r-RI, and the remaining RNAase activity was determined using UpA as the substrate. Fig. 2(a) shows that under the standard assay conditions (Vicentini *et al.*, 1990) a sigmoidal curve was obtained. It is noteworthy that full-length r-RI (peak 2) yielded a normal titration curve under these conditions (Fig. 2a). These results could be explained by assuming that a constant amount of Δ r-RI adsorbed to the polypropylene vessel, and that, therefore, the percentage of inhibitor lost would be larger at lower concentrations of protein. When 0.02% (w/v) Tween 20 was included in the assay buffer, a normal titration curve was observed (Fig. 2a). To determine the kinetic constants for the inhibition of RNAase by Δ r-RI, the effects of various concentrations of Δ r-RI on the rate of UpA hydrolysis were determined. The data shown in Fig. 2(b) could be fitted, by non-linear regression (Stone & Hofsteenge, 1986), to the equation for slow, tight-binding, inhibition (Morrison & Stone, 1985; Morrison & Walsh, 1988). This analysis yielded estimates for the apparent inhibition constant (K_i') and the apparent association rate constant (k_{on}'). The value of K_i' was corrected for the presence of substrate, whereas k_{on}' , at the substrate concentration used, closely approximated to the true value of k_{on} (Vicentini *et al.*, 1990). The value for k_{off} was calculated from the relationship $K_i = k_{off}/k_{on}$. In Table 2 the values of these constants are compared with those obtained for r-RI.

DISCUSSION

The finding that Δ r-RI could bind to RNAase A and inhibit its activity was unexpected, considering the results of Lee & Vallee (1990a). Using deletion mutagenesis, these workers found that removal of residues 1–62 or 30–86 of human placental RI destroyed its RNAase-inhibitory activity. Given the high degree of sequence identity (78%) between human and pig RI in these regions, a similar result would have been expected with Δ r-RI. The most likely explanation for this apparent inconsistency seems to be the altered surface properties of RI with deletions in the *N*-terminus. Removal of residues 1–90 (or 1–93) of RI was found to yield a molecule that adsorbed to plastic surfaces more readily than did full-length RI. In the absence of a surfactant (Tween 20) we also observed little inhibition of RNAase at low concentrations of Δ r-RI (Fig. 2a).

The results obtained with Δ r-RI indicated that residues 1–90 (or 1–93) can be removed from pig RI with a minimal decrease in its affinity for RNAase. In fact, the binding energy only decreased by 2% (Table 2). It can be concluded that this part of the molecule contributes little to the binding energy. Residues 144–257 and 315–371 of (human) RI can also be deleted without destroying the inhibitory activity (Lee & Vallee, 1990a,b).

Fig. 3. Schematic representation of the structure of the RI molecule

The areas of RI that can be deleted without destroying its RNAase-inhibitory activity are shown in relation to the repeating units of the molecule. The portion of the molecule containing the repeats is shown as a wide bar with the numbering indicated above it. *N*- and *C*-terminal extensions are shown as narrow bars. ▨, Residues 144–257 or 315–371 (see the text) were deleted from human RI by Lee & Vallee (1990a,b); ▩, residues 1–90 (or 1–93); the present study.

(Human RI contains a five-amino-acid *N*-terminal extension compared with pig RI. Thus the corresponding residues in the human sequence have numbers that are 5 larger than those given in Fig. 3.) Assuming that this finding also holds for pig RI, only three relatively small areas in the primary structure remain as candidates for the sites that mediate RNAase inhibition (Fig. 3).

An interesting property of the RI–RNAase complex is that its stability is dependent on the presence of reducing agents in the medium. Moreover, the complex can be dissociated by modification of RI with thiol-blocking reagents, for example pHMB and *N*-ethylmaleimide (Roth, 1958; Shortman, 1962; Blackburn *et al.*, 1977). Pig RI contains 30 cysteinyl residues (Vicentini *et al.*, 1990), and it is not known which of these mediate the thiol-dependence. Since RI containing any of the three deletions mentioned above can still be eluted from RNAase-Sephadex by pHMB, it appears that these areas do not contain the sensitive cysteinyl residue(s). Further studies are required to identify these remaining residues in the remaining portions of the molecule.

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