One functional subunit is sufficient for catalytic activity and substrate specificity of *Escherichia coli* endoribonuclease III artificial heterodimers

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Received 1 February 2002; revised 15 March 2002; accepted 15 March 2002

First published online 10 April 2002

Edited by Thomas L. James

Abstract To study the intersubunit communication required for the activity of the normally homodimeric enzyme endoribonuclease III of *Escherichia coli* we have constructed and analysed an artificial heterodimer. This heterodimer is composed of one wild-type and one catalytically inactive subunit. The inactive subunit has one amino acid exchanged (E117K, *rnc70* mutant) which abolishes cleavage activity but still allows substrate binding of a *rnc70*-homodimer. Our results show that one functional active site is sufficient for cleavage activity of the heterodimer. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: RNase III; Heterodimer; RNA processing; Dimeric enzyme

1. Introduction

Endoribonuclease III (RNase III) of *Escherichia coli* is a homodimeric enzyme composed of two 25.6 kDa (226 amino acids) subunits [1,2]. Until now it has not been analysed if the active sites of the two subunits of the enzyme can function independently of each other or if their catalytic activity shows interdependence. RNase III shows a specificity for double-stranded RNA (dsRNA) and is involved in various steps of rRNA, mRNA, small nuclear RNA and small nucleolar RNA processing in pro- and eukaryotic organisms [3,4]. Recently it has been shown that RNase III enzymes, namely the eukaryotic 'dicer', are key players in the phenomenon of RNA interference or posttranscriptional gene silencing [5,6]. RNase III is a phosphodiesterase and its RNA cleaving activity depends on divalent metal ions, preferably Mg^{2+}. RNA cleavage products possess 5′-phosphate and 3′-hydroxyl termini showing a 3′-overhang of two nucleotides [7]. Under suboptimal or unphysiological conditions (low salt, Mn^{2+}) the enzyme cleaves an RNA substrate at non-canonical ('secondary') sites in addition to the primary (canonical) sites [1,8–11].

Just recently the crystal structure of RNase III from *Aquifex aeolicus* has been solved by X-ray analysis [12]. The structural data indicate that *A. aeolicus* RNase III might contain two RNA-cutting sites within each of the two active centres. This observation points in the direction that with certain substrates four cleavages per dimer could occur.

Bacterial RNase III consists of an N-terminal catalytic domain and a C-terminal dsRNA binding domain (dsRBD). The domains function independently of each other and it has been noted that the N-terminal part lacking the dsRBD still forms a dimer [13–15]. The N-terminal part seems to be the major determinant of cleavage specificity, although the dsRBD does contribute to site selection in some cases and facilitates substrate binding [13,15].

To address the question of intersubunit communication we constructed artificial heterodimers of *E. coli* RNase III. One of the subunits is derived from a catalytically inactive form of RNase III, the so-called *rnc70* mutant (E117K), where glutamic acid (E) at position 117 has been exchanged for lysine (K) [14]. This mutant is still able to bind RNA but lacks cleavage activity. In our experiments, the formation and purification of heterodimers was achieved by coexpression and subsequent two-step affinity chromatography of the associated subunits (Fig. 1). As indicated in Fig. 1, three different RNase III species are likely to occur during coexpression in vivo: (1) a (hexahistidine (His))6-homodimer of approximately 54 kDa, which represents the mutant RNase III and should be catalytically inactive. (2) A glutathione *S*-transferase (GST)-homodimer of about 103 kDa representing the wild-type (wt) RNase III. (3) A heterodimeric RNase III of approximately 78 kDa composed of one mutant (Rnc70) and one wt subunit. After purification of the artificial heterodimers we used in vitro transcribed RNAs as substrates for the different RNases III to determine the contribution of the enzyme's individual subunits.

2. Materials and methods

2.1. Bacterial strains

E. coli strains used for plasmid propagation and protein expression were JM109 (NEB), XL1-Blue (Stratagene), and BL21(DE3) (Invitrogen).

2.2. Construction and expression of recombinant RNases III

The sequences of the polymerase chain reaction (PCR) primers used for amplification of the *E. coli* wt *rnc* and the mutant *rnc*70 genes were as follows (restriction sites underlined): *Bam*-*rnc*-hetero: 5′-CGCGGATCCAACCCCATCGTAATT-3′, and *Sal*-*rnc*-hetero: 5′-ACGCGTCGACTCATTCCAGCTCCAGTTT-3′. As templates we used genomic DNA from *E. coli* JM109 for the wt *rnc* gene and the plasmid pACS701 [14] for the *rnc*70 gene, respectively. The resulting PCR products were cut with *Bam*HI and *Sal*I and cloned into the corresponding sites of the polylinker of the pHisRVK hexahistidine-tag vector [17] and pGEX-4T-1 GST-tag plasmid (Amersham-Pharmacia), respectively. From the pHisRVK plasmid the gene for *Eco*RV had been cut out previous to the insertion of the *rnc* gene. For protein expression both vectors were cotransformed and propagated in *E. coli* BL21 cells at 37°C using standard I medium (Merck) containing am-

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PII: S0014-5793(02)02653-4

picillin (200 μg/ml) and kanamycin (25 μg/ml). Coexpression of the RNase III subunits was induced by adding isopropyl β-D-thiogalactopyranoside at a final concentration of 1.5 mM when cells reached an OD_{600} of 0.8. After continued incubation at 37°C for 2 h, the cells were harvested by centrifugation at 5000×*g* and stored at −70°C.

2.3. Purification of heterodimers

For purification of RNase III heterodimers (*E. coli* wt RNase III/*rnc*70 mutant) two subsequent affinity chromatography steps were carried out [16,17] (Fig. 1). As a first step we used binding of the recombinant proteins to glutathione Sepharose (Amersham-Pharmacia). In brief, for purification of GST-tagged RNase III species (i.e. heterodimer or wt RNase III) 4 g cells overexpressing the enzyme were resuspended in 40 ml cold phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, pH 7.3, 0.01% Lubrol), incubated for 30 min on ice and disrupted by sonication. 20% Triton X-100 was added to a final concentration of 1%. The samples were shaken for 30 min at 4°C and then centrifuged for 10 min at 12 000×*g* and 4°C. The supernatant was then used for binding to 4 ml glutathione Sepharose 4B. The samples were shaken for 30 min at room temperature, packed into a column and washed with 20 volumes of cold PBS. For elution, one volume of freshly prepared buffer containing reduced glutathione was used.

As a second step for purification of RNase III heterodimers binding to Ni-NTA agarose (Qiagen) was performed (Fig. 1). The Ni-NTA agarose was washed with 20 volumes of buffer containing 50 mM Tris–HCl, pH 7.5, 250 mM NaCl and 50 mM imidazole [18]. The heterodimers were eluted with buffer containing 130 mM imidazole. Alternatively, 3 g cells overexpressing the RNase III-*rnc*70 were used for Ni-NTA agarose purification of the inactive homodimer.

2.4. In vitro transcription of RNAs and enzymatic assays

As template for the synthesis of the N26 RNA we used an oligonucleotide with an annealed 18-mer promoter oligonucleotide [11]. In vitro transcription and purification of the internally labelled transcript were performed as previously described [19,20].

Cleavage assays (5000 cpm of RNA pro-assay) were performed in a cleavage buffer (30 mM Tris–HCl, pH 7.5; 10 mM $MgCl_2$, 130 mM KCl, 5% glycerol) at 37°C. Changes of the standard conditions are indicated. Reaction products were incubated at 65°C for 3 min with formamide-containing dye and analysed on a 10% PAA/7 M urea gel. Bands were detected and quantified using a BioRad molecular imager and the Quantity One (BioRad) software.

2.5. Protein crosslinking with glutaraldehyde and size exclusion chromatography

To monitor the composition of the different dimeric RNase III variants by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE; see Fig. 4) we used a crosslinking procedure that has been described for yeast RNase III [21]. Glutaraldehyde has been used for crosslinking of RNase III subunits which can subsequently be detected in SDS–PAGE as holoenzymes because the crosslink avoids disruption of the dimer during electrophoresis.

An increasing concentration of freshly diluted glutaraldehyde was added to an RNase III solution to a final concentration of 0.05, 0.1, 1 and 2%, respectively. The samples were incubated for 10 min at 30°C and then analysed on a SDS–PAGE gel (10%; AA/BAA, ratio 80:1). Electrophoresis took place at ∼12 mA overnight.

In order to test for the presence of dissociated monomers in the GST-tagged wt RNase III preparation, the fraction was applied in 50 mM Tris–HCl, pH 7.0, 200 mM KCl to a HiLoad™ Superdex 75 column. A fast performance liquid chromatography System (Pharmacia) was used for the analysis adjusted to a flow rate of 1.5 ml/min.

3. Results

3.1. Purification of proteins

The heterodimers have been purified to apparent homogeneity. On a denaturing SDS–PAGE both subunits, containing either a hexahistidine- or a GST-tag, were present in an equivalent amount (Fig. 2A). The concentration of the purified heterodimer was in the range of 20 ng/μl.

In order to test whether the hexahistidine- or GST-tagged

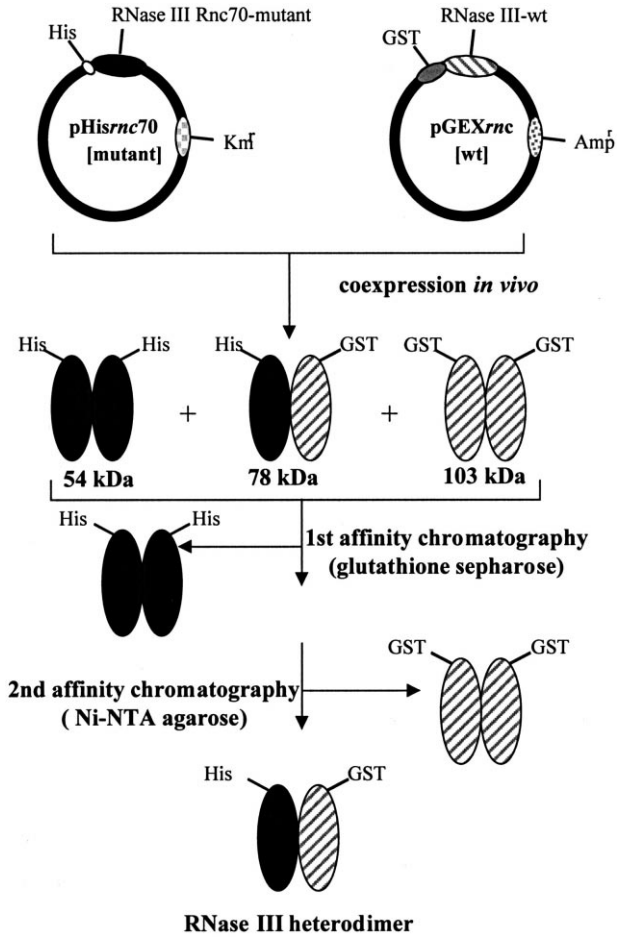

Fig. 1. Schematic overview of the steps used in coexpression and purification of the RNase III heterodimer. GST: glutathione *S*-transferase tag. His: hexahistidine tag. Sizes of the different RNases III are indicated in kDa.

subunits were really combined to heterodimers, glutaraldehyde was used to stably link the two subunits of the homodimeric and heterodimeric enzymes which subsequently have been analysed by SDS–PAGE (Fig. 2B). After silver-staining of the gel we could detect bands corresponding to the sizes of the three possible crosslinked RNase III species. The expected sizes were approximately 54 kDa for the (His)6-homodimer, 78 kDa for the heterodimer and about 103 kDa for the GST-homodimer. The GST-homodimer and the heterodimer show a running behaviour which is slower than expected from their calculated sizes. This is most likely due to the crosslink. The additional bands at 31 kDa in lanes where the GST-homodimer was loaded represent degradation products of RNase III and the GST-tag which could also be observed on other gels (not shown). The observed smear for the bands corresponding to the crosslinked RNase III species often occurs during electrophoresis of proteins which have been crosslinked by glutaraldehyde and has also been reported by other authors (e.g. [21]). Due to the purification procedure of the heterodimer, no RNase III monomers are present in these fractions. To test for the presence of dissociated monomers in the GST-tagged wt RNase III preparation, we analysed the fraction by gel filtration. Repeated experiments revealed that less than 5% of the enzyme are present as monomers (not shown).

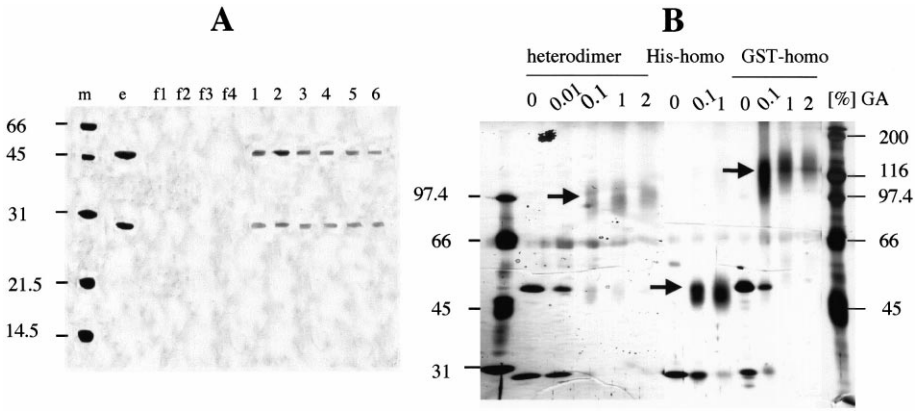

Fig. 2. A: Silver-stained SDS–PAGE showing the purified subunits of the heterodimeric RNase III after glutathione Sepharose and subsequent Ni-NTA chromatography. m: protein marker, sizes are indicated in kDa. e: eluate from the previous glutathione Sepharose chromatography (first column). f1–f4: eluate from the washing procedure. 1–6: fractions of the heterodimer after elution of the Ni-NTA column (second column). B: Silver-stained SDS–PAGE showing different RNase III species which have been crosslinked by glutaraldehyde (GA). The amount of glutaraldehyde used is indicated above each lane (in % GA). Protein markers were loaded on the first and last lane, molecular weights are indicated in kDa. The different RNase III dimeric proteins are indicated by arrows.

3.2. RNA degradation assays

To test the enzymatic activity of the purified RNases III we used an in vitro transcribed RNA as substrate, which has been described previously [11] (Fig. 3A). We first incubated identical amounts of the two homodimers and the heterodimer (50 ng) with the RNA substrate under different salt conditions. It is known that the concentration of KCl influences the cleavage activity and the specificity of RNase III [1,9,10]. We found that the GST-homodimer composed of the catalytically active subunits and the heterodimer cleave the N26 substrate at identical positions (Fig. 3). Cleavage at the secondary site is enhanced at low salt concentration for both enzymes. The heterodimer was more sensitive to high salt concentrations. Our data also confirmed that the (His)6-homodimer composed of the catalytically inactive subunits is unable to cleave the N26 substrate in the presence of 130 mM KCl and showed very low cleavage activity at 50 mM KCl or below (Fig. 3).

We then incubated the N26 RNA substrate with different amounts of either the GST-homodimer or the heterodimer. Under all concentrations tested the two enzymes showed identical cleavage patterns and similar relative activities (Fig. 4A,B).

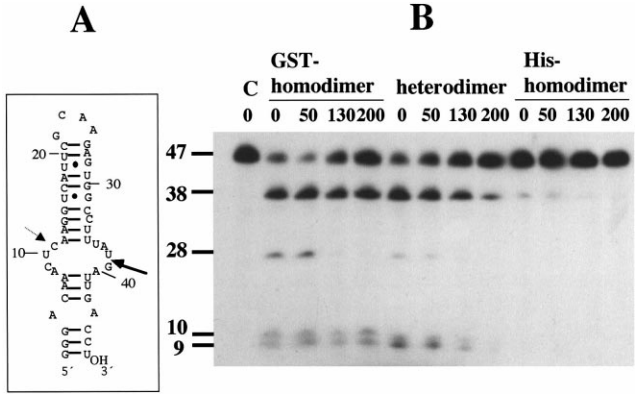

Fig. 3. A: The N26 RNA substrate with the primary and secondary cleavage sites indicated. B: Cleavage of the N26 substrate by the GST-homodimer, the heterodimer and the $(His)_6$-homodimer at increasing concentrations of KCl. 20 nM N26 substrate was incubated for 3 min with 50 ng of each protein fraction. C indicates the control which was incubated without the addition of enzyme. The numbers above the lanes indicate the concentration of KCl in mM.

4. Discussion

Many enzymes act as dimers of identical subunits. In some cases the active site is composed of amino acid residues from both subunits. In other enzymes the active sites are formed by amino acid residues from one subunit, but subunit association

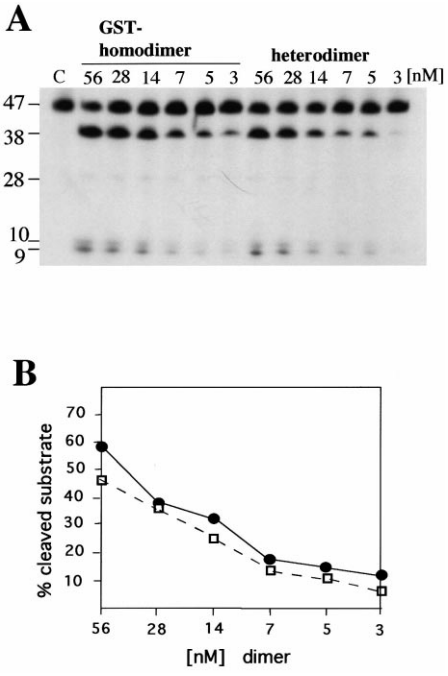

Fig. 4. A: N26 RNA incubated for 3 min at 37°C with different amounts of *E. coli* RNase III GST-homodimer or RNase III heterodimer, respectively. The concentration of enzyme (nM dimer) incubated with 20 nM substrate for 3 min is indicated above each lane. Sizes of the RNA fragments are indicated in nucleotides (nt) on the left site of the gel. B: Percent of substrate cleaved by the homodimeric (●) or heterodimeric (□) RNase III, respectively, as calculated by quantification of the total activity per lane in relation to the radioactivity of all product bands.

leads to activation. Many enzymes acting on double-stranded nucleic acids have a dimeric structure. For most of these enzymes it is not known whether the dimeric structure is required for binding, for catalytic activity, or for both.

In order to study the interdependence of the two subunits of the RNase III dimer during RNA cleavage we created and analysed a heterodimer of one wt subunit and one subunit stemming from the catalytically inactive *rnc70* mutant. Crosslink experiments confirmed that after co-expression of the two proteins and purification of the heterodimer by two consecutive affinity chromatographies most of the dimers exist as heterodimers, indicating that the rate of dissociation/reassociation of the two subunits is very low. Of course we cannot completely exclude that a small percentage ($< 10\%$) of active homodimers is formed in this fraction which cannot be detected on gels.

Our cleavage experiments using the N26 substrate show that *E. coli* RNase III can function with only one catalytically active subunit. A mechanism for RNase III catalysed cleavage was recently proposed on the basis of the structural analysis of *A. aeolicus* RNase III [12]. The structural data suggest that the fold of the polypeptide chain and its dimerisation creates a valley with two compound active centres at each end of the valley. In the *Aquifex* enzyme the active centre is formed by the acidic side chains of amino acids E37, E40, D44 of the signature box, amino acids D107, and E110, and the juxtaposed E64 acidic side chain of the other subunit. These residues correspond to E38, E41, D45, E114, E117 and E65 of the *E. coli* enzyme. The point mutation *rnc70* changes the E117 codon to a lysine codon. Thus, the heterodimer we analysed should harbour one active centre composed of the amino acids E38, E41, D45, E114 and E117 stemming from the wt subunit and E65 stemming from the mutant subunit, while the other centre is inactive due to the lack of E117.

When we used equal molar amounts of the homodimer and the heterodimer, the heterodimer was able to cleave almost the same amount of the N26 substrate as the homodimer. Therefore, we can exclude that the observed cleavage is due to a minor contamination of the fractions with active homodimer. Gel filtration analysis revealed that the activity of the GST-tagged wt RNase III preparation is not reduced by the presence of a considerable amount of dissociated monomers. A His-tagged wt RNase III showed similar activity when compared to a wt enzyme without tag [22]. Thus, the presence of the His-tag in the heterodimer should not significantly influence its activity, i.e. by facilitating RNA binding.

If an RNA is positioned in the valley of the RNase III dimer but can only be cleaved by one of the two active centres at a certain site, one would expect that only 50% of the substrate molecules can be cleaved when only one active centre is present. The fact that the heterodimer shows more than 50% of the relative activity of the wt enzyme indicates that both active centres may be able to cleave the N26 substrate at the primary site. Alternatively, the asymmetric heterodimer may bind to the RNA substrate with high preference for one of the two possible orientations. In any case the data show that only little interdependence between the two composite active centres exists.

The construction and analysis of additional heterodimers composed of two subunits with different mutations could allow further testing of the contribution of individual amino acids to the functionality of the active sites in the future.

Acknowledgements: We would like to thank Wolfgang Wende and Alfred Pingoud for providing the plasmid pHis and for helpful comments, the laboratory of Donald Court for providing the plasmid encoding the mutant (Rnc70) RNase III, and Steffen Wagner for assistance in enzyme purification. This work was supported by the Deutsche Forschungsgemeinschaft (DFG Kl 563/11-1) and the Justus-Liebig-University Giessen.

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