The RNA N-Glycosidase Activity of Ricin A-chain

THE CHARACTERISTICS OF THE ENZYMATIC ACTIVITY OF RICIN A-CHAIN WITH RIBOSOMES AND WITH rRNA*

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Ricin A-chain cleaves the N-glycosidic bond at A-4324 in 28 S rRNA when intact rat ribosomes are the substrate. Cleavage occurs at a concentration of the toxin of 1 × 10^{-10} M, and specificity for this single residue is retained when the concentration is as high as 3 × 10^{-7} M. The apparent Michaelis constant (K_m) for the reaction is 2.6 μM, and the turnover number (K_cat) is 1777 min^{-1}. The same N-glycosidic bond is cleaved by ricin A-chain in naked 28 S rRNA, but at a greatly reduced rate. The K_m value for this reaction is 5.8 μM. The results suggest that the A-chain has a similar affinity for 28 S rRNA in ribosomes and in the absence of ribosomal proteins. Ricin A-chain has no effect on 23 S rRNA in Escherichia coli ribosomes, however, the N-glycosidic bond at A-2600 in naked 23 S rRNA is cleaved by the toxin; this corresponds to the ricin site in eukaryotic 28 S rRNA. Since the K_m value (5.8 μM) for the reaction with E. coli 23 S rRNA approximates that obtained with rat liver ribosomes, it is possible that E. coli ribosomal protein(s) protect this site against ricin attack in intact ribosomes. Ricin A-chain also acts on naked 16 S rRNA cleaving the N-glycosidic bond of adenine at position 1014. The results suggest that ricin A-chain recognizes a specific structure in rRNA, perhaps a loop and stem having the sequence GAGA in the loop.

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Figure 1: Effect of ricin A-chain on rat ribosomes and on rRNA. In A, rat liver 80 S ribosomes (38 μg) in 25 μl of Buffer A (25 mM Tris/HCl, pH 7.6, 25 mM KCl, 5 mM MgCl₂) and, in B, naked rRNA (21.9 μg) in 25 μl of Buffer B (25 mM Tris/HCl, pH 7.6, 100 mM NaCl, 10 mM MgCl₂) were incubated at 37 °C with ricin A-chain in the concentrations indicated. After 10 min for ribosomes or 60 min for naked rRNA, the nucleic acids were extracted from the reaction mixture with phenol and sodium dodecyl sulfate. The RNA (5 μg) was treated with aniline at acidic pH at 60 °C for 10 min to induce scission of the phosphoribose chain at an apurinic site. The samples were analyzed by electrophoresis in 2.5% polyacrylamide, 0.5% agarose composite gels. The RNA was stained with ethidium bromide. The arrowsheads designate the R-fragment and the arrows the S-fragment.

Figure 2: Cleavage of the N-glycosidic bond in the large rRNA as a function of the concentration of ricin A-chain and of the time of the reaction. In A, 3²P-labeled 80 S ribosomes (114 μg; 1.5 × 10⁶ cpm/mg of RNA) were incubated for 10 min at 37 °C in 25 μl of Buffer A with the concentration of ricin A-chain indicated. The RNA was extracted and separated on composite gels after chain scission with aniline. The radioactivity in the R-fragment and in the 5.8 rRNA bands was determined. The extent of the cleavage of the N-glycosidic bond was calculated from the molar ratio of 5.8 S rRNA to the R-fragment. In B, 3²P-labeled total rRNA from rat ribosomes (63.4 μg; 1.5 × 10⁶ cpm/mg, ○—○) or 3²P-labeled total rRNA from E. coli ribosomes (39.1 μg; 1.7 × 10⁶ cpm/mg, X—X) were incubated for 30 min in 25 μl of Buffer B. The extent of the cleavage of the N-glycosidic bond was determined as above. In C, each substrate was incubated for the indicated period of time with ricin A-chain; the concentration of the toxin was 1 × 10⁻⁸ M for 80 S ribosomes (○—○) and 5.0 × 10⁻⁸ M for naked rat liver rRNA (●—●) and E. coli rRNA (X—X). The extent of the cleavage of the band at each time interval was determined.

Treatment of the 553-fragment with A-chain made the phosphodiester bonds at G-4323 and at A-4324 resistant to the action of ribonucleases. Thus, the A-chain acts on the 553-fragment to cleave the N-glycosidic bond at the same site as in ribosomes. These results suggest that the A-chain recognizes a specific structure in the RNA. In addition, it is apparent that removal of ribosomal proteins decreases the sensitivity of this bond to the toxin. Thus, r-protein(s) modulate the response to the toxin.

Preparations of 3²P-labeled 80 S ribosomes or of naked rRNA, at concentrations of 0.5 to 2.7 μM, were treated with ricin A-chain, followed by aniline, and the radioactivity in the R-fragment was determined (cf. "Experimental Procedures"). These steady state kinetic experiments were performed so that it was the initial velocity of the reaction that was assessed. From a double reciprocal plot of the data (Fig. 4), we determined that the maximum velocity (V_max) for ricin A-chain with ribosomes is 10.5 μM/min and the apparent Michaelis constant (K_M) is 2.6 μM. The turnover number (K_cat)
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Effect of Ricin A-chain on Naked rRNAs from E. coli Ribosomes—Ricin A-chain does not inactivate prokaryotic ribosomes (1) nor does it cleave an N-glycosidic bond in the RNA of these ribosomes (3). However, the reason the A-chain is without effect on E. coli ribosomes is not apparent insomuch as 23 S rRNA contains a sequence homologous to the ricin site of rat 28 S rRNA. In an attempt to solve this problem, total rRNA was isolated from E. coli ribosomes and was incubated with varying amounts of ricin A-chain (Fig. 5). An analysis of the RNA by gel electrophoresis, after the treatment of the sample with aniline, gave an unexpected result. Ricin treatment of the naked RNA led to the cleavage of N-glycosidic bonds at more than one site as is apparent from the production of four fragments rather than two (Fig. 5, lanes 3–8, bands designated a, b, c, d). These bonds are not affected by the A-chain when the RNAs are in ribosomes (Fig. 5, lane 1) which suggests that r-protein(s) act, in some way, to protect the rRNA from ricin attack. The A-chain at a concentration of 2 × 10−6 M was effective in producing fragments a and d, and, even if the concentration was increased 75-fold, no more than four fragments were formed. The formation of the fragments from 23 S rRNA was dependent on the concentration of the A-chain (Fig. 2B) and on the incubation time (Fig. 2C), and the kinetics were similar to those observed with 28 S rRNA. The amounts of fragments b and c appeared to be less than that of fragments a and d (Fig. 5). Fragments a and d were derived from 23 S rRNA and fragment b and c from 16 S rRNA; formation of the fragments was dependent on treatment with aniline (Fig. 5, lanes 10–13).

The sites of cleavage of 23 S and 16 S rRNAs by ricin A-
Fig. 5. Effect of ricin A-chain on naked E. coli rRNA. Total rRNA was extracted from E. coli ribosomes (strain K-A19) with phenol and sodium dodecyl sulfate. Samples (22.8 μg) were incubated in 25 μl of Buffer B at 37 °C for 60 min with concentrations of the A-chain indicated. Ribosomal particles were incubated in the same way but in Buffer A. The RNA was analyzed as described in the legend to Fig. 1. In lanes 3–8 the substrate was naked rRNA. In lane 1, the substrate was ribosomes. In lanes 10 and 11, preparations of 23 S rRNA, and, in lanes 12 and 13, 16 S rRNA, were analyzed after treatment with ricin A-chain, but either before (lanes 10 and 12) or after (lanes 11 and 13) exposure to aniline. In lanes 2 and 9, rRNA from α-sarcin-treated ribosomes was analyzed; the α-fragment serves as a marker. (2). The letters a, b, c, and d designate the fragments produced by the action of ricin A-chain.

Fig. 6. Determination of the sequence of nucleotides in fragments from ricin-treated E. coli rRNA. Fragments a and b produced by ricin treatment of E. coli rRNA (cf. Fig. 5) were made radioactive at the 5' termini with 32P]ATP and T4 kinase. The fragments were repurified by electrophoresis in 3.5% polyacrylamide gels and were partially digested with ribonuclease T1 (G), ribonuclease U2 (A), or ribonuclease PhyM (A/U). Alkaline digests of the fragments were repurified by gel electrophoresis in 20% polyacrylamide gels. In A, fragment a and the α-fragment were analyzed; in B, fragment b was analyzed. Nucleotides are numbered from their 5' terminus (20).

chain were determined. The fragments a and b were isolated by sucrose gradient centrifugation, and each 5' terminus was labeled with [γ-32P]ATP after treatment with phosphatase. Each of the radioactive fragments was repurified by gel electrophoresis, and the 5'-terminal nucleotide sequences were determined by an enzymatic method. The 5'-end of fragment a is G-2661 which is adjacent to the α-sarcin site (Fig. 6A). α-Sarcin cleaves the phosphodiester bond between G-2661 and A-2662 of E. coli 23 S rRNA in ribosomes. This is the first direct determination of the α-sarcin cleavage site in E. coli 23 S rRNA. The results indicates that ricin A-chain cleaves the N-glycosidic bond at A-2660 of 23 S rRNA since the β-elimination reaction catalyzed by aniline at acidic pH resulted in chain scission on the 3' side of A-2660 giving G-2661 as the 5'-end of fragment a. This residue (A-2660) corresponds to A-4324 in rat 28 S rRNA. In a similar manner, the site of action of ricin A-chain on 16 S rRNA was identified as A-1014 (Fig. 6B). Fragment c is derived from the 5'-end of 16 S rRNA (data not shown).

The Kcat value for the action of ricin A-chain on E. coli 23 S rRNA is 3.3 μM, which is close to the value obtained with rat ribosomes (Table I). The Kcat for the reaction with 23 S rRNA is close to that observed with naked 28 S rRNA as the substrate. The results indicate that 23 S and 28 S rRNAs contain an equivalent structure for ricin action.

The finding that ricin A-chain cleaved the N-glycosidic bond of A-1014 in 16 S rRNA was unexpected. This, however, provided the opportunity to derive a consensus structure for
the site of ricin attack on rRNA. The secondary structures proposed for the ricin-sensitive sites in rat 28 S, yeast 26 S, and E. coli 16 S and 23 S rRNAs were examined (Fig. 7A). It appears that the ricin A-chain acts on an adenosine residue in the sequences GAGA present in a loop with a stem of 6 or 7 base pairs in length (Fig. 7A). There are a number of such loop and stem structures in rRNA. These structures are presented in Fig. 7B. From the comparison of the structures in A and B, we propose that ricin A-chain acts on the first adenosine residue in the sequence of GAGA in a loop with a stem of 6 or more base pairs. In addition, the location of that adenosine residue in the loop seems to be another requirement for the A-chain action.

**DISCUSSION**

Direct measurement of the $K_m$ and $K_{cat}$ for ricin A-chain yielded values of 2.6 $\mu$M and 1777 min, respectively. These values are in agreement with those reported by Olnes et al. (11) who used an indirect method. The $K_m$ we obtained accounts for the extreme toxicity of ricin since the intracellular concentration of ribosomes is of the order of 1 $\mu$M (20).

It is remarkable that the $K_m$ for ricin A-chain with naked 28 S rRNA is the same as that for ribosomal particles. This suggests that the A-chain is able to bind to the same site on naked 28 S rRNA as in ribosomes and with a similar affinity. The interpretation is in fact substantiated by the direct measurement of the dissociation constant ($K_d$). Hedblom et al. (12) have shown that 1 molecule of ricin binds to one rat liver ribosome with a $K_d$ of 3 $\mu$M. Thus, ricin A-chain appears to recognize a specific structure in rRNA and the recognition does not seem to require protein. Moreover, intact 28 S rRNA is not essential since the 553-nucleotide 3' fragment that contains the ricin site can serve as a substrate for the toxin. Eukaryotic ribosomal protein(s) may condition ricin action at a step after binding since their removal results in a large reduction in the $K_{cat}$.

Ricin A-chain cleaves an N-glycosidic bond in E. coli 23 S rRNA only in the absence of r-proteins. The site of action of the A-chain is A-2600, adjacent to the $\alpha$-sarcin site. The $K_m$ and the $K_{cat}$ for the reaction with E. coli 23 S rRNA were almost the same as for rat 28 S RNA supporting the assumption that the A-chain recognizes a specific structure in the RNA. It is important that the A-chain does not act on 23 S rRNA in ribosomal particles. This suggest that prokaryotic r-protein(s) in some way interfere with the binding of the A-chain. Indeed, Hedblom et al. (12) have shown directly that ricin A-chain does not bind to E. coli ribosomes.

An unexpected finding was that ricin A-chain also cleaves an N-glycosidic bond at position A-1014 in 16 S rRNA. This leads to the proposal that it is the tetranucleotide sequence, GAGA, in a loop with a stem of 6 or 7 base pairs that is recognized by ricin. Recently, Montfort et al. (21) have presented a three-dimensional structure for ricin A-chain derived from crystallographic analysis. They have found a cleft in the molecule and have suggested that it is the active center of the protein. This cleft may accommodate the recognition structure we have proposed during the cleavage reaction.

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