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# α-SARCIN CLEAVES RIBOSOMAL RNA AT THE α-SARCIN SITE IN THE ABSENCE OF RIBOSOMAL PROTEINS

Stephen P. Miller and James W. Bodley

Department of Biochemistry
University of Minnesota
Minneapolis, Mn. 55455

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**SUMMARY:** α-Sarcin is capable of specifically cleaving the single phosphodiester bond in the "α-sarcin site" of both *Escherichia coli* and *Saccharomyces cerevisiae* large rRNAs in the absence of ribosomal proteins. With both sources of rRNA, the rate of cleavage was comparable with and without ribosomal proteins but more complete cleavage was observed in the absence of ribosomal proteins. These observations contrast with earlier findings and indicate that ribosomal proteins are not essential to the unique specificity of the cleavage of rRNA by α-sarcin. 

α-Sarcin is a cytotoxic protein which possesses an unique ribonuclease activity. The cytotoxicity of the protein is the result of the inactivation of the ribosomes (1,2) through the cleavage of a single phosphodiester bond in the rRNA of the large subunit (3-5). The site of cleavage, the "α-sarcin site", is within a highly conserved purine-rich sequence which is present in the large rRNAs of prokaryotic 70S ribosomes, eukaryotic 80S ribosomes, and the ribosomes of mitochondria and chloroplasts (5). The resulting oligonucleotide (termed the α-fragment) cleaved from the 3'-end of the large rRNA is between 240 and 500 nucleotides in length depending on the location of the cleavage site in the RNA. This unique specificity of α-sarcin was observed with intact ribosomes but not with naked rRNA (3-5) suggesting that ribosomal proteins play an important role in creating the α-sarcin site. The inactivation of ribosomes by α-sarcin has brought to light the functional importance of this region of the large rRNA to the interactions of the ribosome with various protein synthesis factors (for a review, see [6]).

The present findings are an outgrowth of work undertaken to determine which ribosomal protein(s) contribute to the specific recognition of rRNA by α-sarcin. In the course of this work we made the surprising observation that partially purified preparations of α-sarcin were capable of recognizing the α-

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sarcin cleavage site in the complete absence of ribosomal proteins. Here we show that the unique specificity of α-sarcin observed with *Escherichia coli* and *Saccharomyces cerevisiae* ribosome substrates can also be seen with their respective naked rRNA substrates.

## MATERIALS AND METHODS

**PREPARATION OF α-SARCIN.** *Aspergillus giganteus* MDH 18894, which was used in the initial isolations of α-sarcin (7), was a generous gift from T. Watson (Michigan Department of Health). Cultures were grown at 30° C in the α-sarcin inducing medium (Medium A) recommended by Olson *et al.* (8). The filtrate obtained from a 72 hr. culture was used as starting material. The method of partial purification employed here was based on the first of two purification steps used in the original isolation of α-sarcin (7). The culture filtrate was passed through a Bio-Rex 70 column (2.5 x 9.0 cm) equilibrated with 50 mM Tris-HCl (pH 7.0) and the column was washed with 50 mM Tris-HCl (pH 7.0), 0.2 M NaCl. α-Sarcin activity was eluted with 50 mM Tris-HCl (pH 7.0), 2.0 M NaCl and the pooled fractions were dialyzed against distilled water. The dialyzed sample was lyophilized and then dissolved in 0.4 M sodium phosphate (pH 6.0) buffer. The partially purified α-sarcin was stored at 4° C and was used in the experiments described below.

**PREPARATION OF RIBOSOMES AND TOTAL rRNA AS SUBSTRATES.** *E. coli* strain B/5 (from J.A. Fuchs, University of Minnesota) was grown at 30° C in LB broth (9). Ribosomes were prepared as previously described (10) except the high salt wash was omitted. The ribosomes were stored at -70° C at a concentration of 5 mg/ml in 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.5 mM EDTA (Buffer A). Total rRNA was prepared from ribosomes (11) by denaturing with 1.0% sodium dodecylsulfate in 50 mM Tris-HCl (pH 7.4) and extracting three times with phenol. After precipitation with ethanol, the rRNA was dissolved in Buffer A at a concentration of 3.0 mg/ml and used as substrate.

*S. cerevisiae* SSL204 (12; from D.M. Livingston, University of Minnesota) was grown at 30° C in YM-1 medium (13). Cell lysates were prepared by the method of Hofbauer *et al.* (14) and used in the preparation of crude unwashed ribosomes (10). Ribosomes were stored at -70° C at a concentration of 5 mg/ml in Buffer A. Total rRNA was prepared from yeast ribosomes as described above for *E. coli*.

**ASSAY OF α-SARCIN ACTIVITY.** The method of Endo and Wool (4) was employed to assay the sensitivity of the substrates to α-sarcin. α-Sarcin was incubated with either ribosomes (50 μg) or total rRNA (30 μg) for 30 min at 37° C in a final volume of 20 μl of Buffer A. The reactions were terminated by the addition of 100 μl of a solution containing 1.0% sodium dodecylsulfate in 50 mM Tris-HCl (pH 7.4). For analysis of the RNA fragments, the RNA was prepared for electrophoresis by phenol extraction followed by precipitation with ethanol. Total rRNA was electrophoresed (15) on 6.0% polyacrylamide-7 M urea gels for *S. cerevisiae* substrates or 7.5% polyacrylamide-7 M urea gels for *E. coli* substrates. The gels were stained with silver (16). Fragments were quantitatively determined and normalized to 5S rRNA by scanning the stained gels at 550 nm using a Beckman DU-8 spectrophotometer.

**SEQUENCING OF THE RNA FRAGMENTS DERIVED FROM THE ACTION OF α-SARCIN ON NAKED rRNA SUBSTRATES.** In order to prepare fragments for 5'-terminal labeling, 2.4 mg of total rRNA extracted from either *E. coli* or *S. cerevisiae* ribosomes (11) was incubated with 1.5 μg of α-sarcin in a final volume of 150 μl as described above. The RNA fragments were separated by polyacrylamide gel electrophoresis and visualized by U.V.-shadowing (17). The excised fragments were eluted from the gel by diffusion and labeled at their 5'-termini with $T_4$ polynucleotide kinase and [$\gamma$-$^{32}$P]ATP (18). The radioactive oligonucleotides were isolated by gel electrophoresis. Enzymatic sequence analysis (18) was performed on 20% polyacrylamide-7 M urea gels, and the gels were autoradiographed overnight.

## RESULTS AND DISCUSSION

Disassembly of *E. coli* ribosomes by the sequential removal of most of the ribosomal proteins by LiCl extraction (19) failed to abolish the specific cleavage of rRNA by α-sarcin (data not shown). These results suggested that the specificity of α-sarcin may not require any of the ribosomal proteins. On the basis of this unexpected finding, we investigated the specificity of α-sarcin action on rRNA substrates in the absence of ribosomal proteins.

Phenol-extracted total rRNA (11) from either *E. coli* or *S. cerevisiae* ribosomes was used as substrate and the fragments produced by α-sarcin were monitored by gel electrophoresis (Fig. 1A and 1B, respectively). For comparative purposes, the rRNA extracted from α-sarcin inactivated ribosomes of either *E. coli* or *S. cerevisiae* is also shown. When ribosomes were the substrate, α-sarcin cleaved the large rRNA at a single phosphodiester bond generating the α-fragment (designated by the arrows) as expected. The

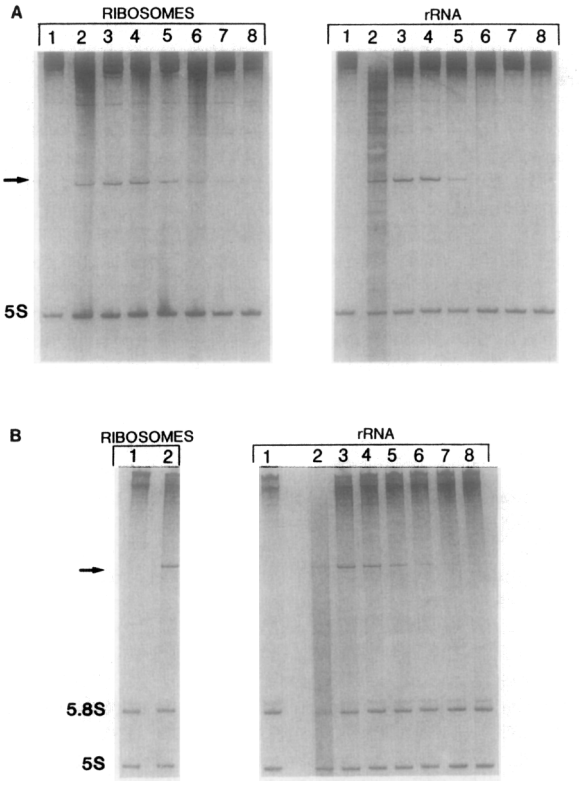


**FIGURE 1. THE EFFECT OF PARTIALLY PURIFIED α-SARCIN ON RIBOSOME AND NAKED rRNA SUBSTRATES.** Ribosomes and total rRNA, isolated from either *E. coli* (Panel A) or *S. cerevisiae* (Panel B), were treated with α-sarcin at the following concentrations: no α-sarcin, lanes 1; 100 μg/ml, lanes 2; 10 μg/ml, lanes 3; 5 μg/ml, lanes 4; 2.5 μg/ml, lanes 5; 1.2 μg/ml, lanes 6; 0.6 μg/ml, lanes 7; and 0.3 μg/ml, lanes 8. Total rRNA was extracted from reaction mixtures and analyzed by polyacrylamide gel electrophoresis as described in "Materials and Methods". rRNA fragments were visualized by silver staining. The positions of the α-fragments are designated by the arrows.

sensitivities of *E. coli* and *S. cerevisiae* ribosomes to cleavage by α-sarcin were comparable. α-Sarcin at a concentration of *ca.* 0.5 μg/ml was sufficient to produce a visible quantity of the α-fragment under the conditions employed here. When either *E. coli* (Fig. 1A) or *S. cerevisiae* (Fig. 1B) naked rRNA was the substrate for α-sarcin, a single cleavage product was also observed. This rRNA fragment was indistinguishable in size from the α-fragment (designated by the arrows) obtained from the rRNA of their respective intact ribosomes. In repeated experiments, no other discrete bands were observed. α-Sarcin at a concentration of *ca.* 0.5 μg/ml was sufficient to generate the fragment and only this single fragment was observed even when the concentration of α-sarcin was increased by two orders of magnitude (Figs. 1A and 1B, rRNA lanes 3-8). At high concentrations of α-sarcin (100 μg/ml), some degradation of all rRNAs was observed (Fig. 1A and 1B, rRNA lanes 2). However, the major discrete product which was observed above the background degradation comigrated with the single fragment observed at lower concentrations of α-sarcin.

The fact that the fragments derived from either ribosomes or their corresponding deproteinized rRNAs were indistinguishable in size suggested that α-sarcin may be catalyzing RNA cleavage at the same site in both substrates. Nucleotide sequences at the 5'-termini of the fragments resulting from the action of α-sarcin on naked rRNA substrates from *E. coli* and *S. cerevisiae* were analyzed to locate the cleavage sites (Fig. 2). The α-sarcin cleavage site within *E. coli* ribosomes has been indirectly deduced by Endo and Wool (4) and confirmed by Hausner *et al.* (20). The 5'-terminal sequence of nucleotides of the fragment derived from *E. coli* naked rRNA was the same as the sequence to the 3'-side of the cleavage site found with intact ribosomes, namely, A-G-G-A-C-C (Fig. 2). Thus cleavage occurs between the guanine residue at position 2661 and the adenine residue at position 2662 of *E. coli* 23S rRNA in the presence or absence of ribosomal proteins. We have previously determined the location of the α-sarcin cleavage site in *S. cerevisiae* ribosomes by direct 5'-terminal sequence analysis of the α-fragment (21). The sequence of nucleotides at the 5'-end of the fragment derived from *S. cerevisiae* naked rRNA was the same, namely A-G-G-A-A-C (Fig. 2). Thus the site of *S. cerevisiae* 26S rRNA cleavage in the presence or absence of ribosomal proteins is the same and occurs between the guanine residue at position 3025 and the adenine residue at position 3026.

Our results clearly demonstrate that α-sarcin, as we have prepared it, is capable of recognizing and catalyzing the cleavage of a single phosphodiester bond within the large rRNAs of both prokaryotes and eukaryotes in the absence of ribosomal proteins. The site of cleavage in both cases is identical to that which occurs in intact ribosomes. The α-sarcin concentration dependence of the cleavage event was comparable in the presence or absence of ribosomal proteins for both sources. However, more complete cleavage by α-sarcin was

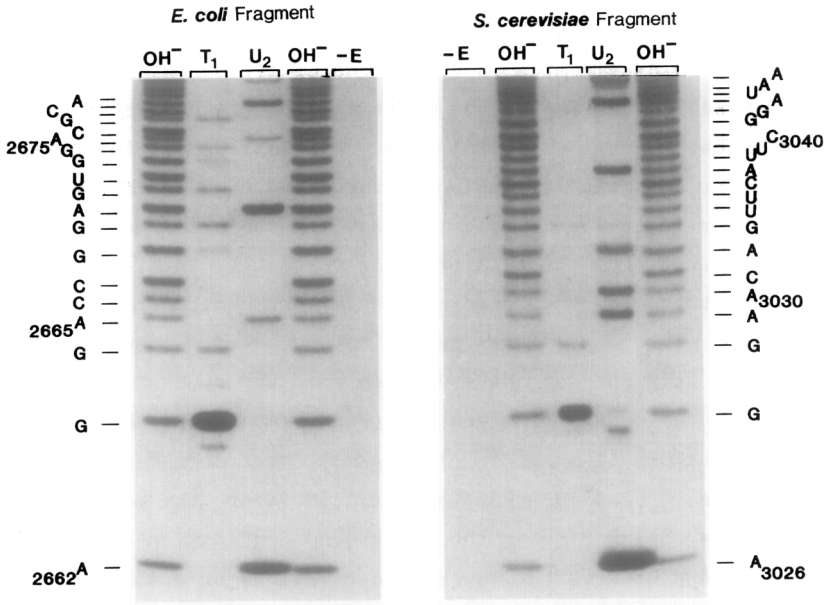


**FIGURE 2. SEQUENCE ANALYSIS OF 5'-TERMINALLY LABELED FRAGMENTS DERIVED FROM THE ACTION OF α-SARCIN ON NAKED rRNA.** Radioactive oligonucleotides from *E. coli* and *S. cerevisiae* α-sarcin treated naked rRNA were partially digested with the G-specific ribonuclease $T_1$ or the A-specific ribonuclease $U_2$. The alkaline digestion ladders are designated $OH^-$ and controls in which the RNA fragments were incubated in the absence of enzyme are designated -E. The respective sequences are shown on the left for *E. coli* and the right for *S. cerevisiae*; purine residues were read from this gel while pyrimidine residues were inferred from the known sequences (4).

consistently obtained in the absence of ribosomal proteins. Cleavage of *E. coli* substrates was approximately 20% and 40% in the presence and absence of ribosomal proteins, respectively, while cleavage of *S. cerevisiae* 26S rRNA in the presence and absence of ribosomal proteins was approximately 30% and 60%, respectively.

The observations reported here are different from those of earlier studies (3-5). Both Schindler and Davies (3) and Endo *et al.* (4,5) observed that specific cleavage of rRNA *required* the presence of ribosomal proteins. In the absence of ribosomal proteins and only at high concentrations of α-sarcin, Endo *et al.* (5) reported a purine-specific endonuclease activity which resulted in the progressive hydrolysis of all rRNAs without detectable preference for the α-sarcin site. Recently, Hausner *et al.* (20) reported nonspecific rRNA degradation as well as limited cleavage at the α-sarcin site in the absence of ribosomal proteins. Differences in the nature of the α-sarcin preparation are a likely cause of the discrepancy between the results reported here and those described earlier. All of the earlier work was performed with a single preparation of α-sarcin which was isolated by Olson *et al.* (7,8) in the middle 1960's. This preparation consisted of a single polypeptide of $M_r$ = 17,000 which existed as a mixture of monomer and dimer in solution (22). The preparation employed in the present study had a specific

activity in the rRNA-cleavage assay, with ribosomes as substrate, similar to that of the earlier preparation but exhibited two polypeptides when analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (data not shown). It would appear that the ability to specifically cleave naked rRNA at the α-sarcin site is a property of the protein which can be lost upon storage or further purification. The nature of this alteration is under study.

The α-sarcin site in rRNA is also acted on by a second class of enzymes which inhibit eukaryotic protein synthesis and is perhaps best typified by the toxin ricin (23,24). These enzymes, which are widely distributed in plants (21,25), act in a most unusual fashion to cleave the N-glycosidic linkage of the adenine residue adjacent to the site of phosphodiester bond cleavage by α-sarcin. As we have shown here for α-sarcin, ricin too is capable of acting on rRNA in the absence of ribosomal proteins, although the reaction of ricin on naked rRNA is much slower and less complete than that on the intact ribosome (23). Clearly, rRNA at the α-sarcin site retains structural features in the absence of ribosomal proteins which allow recognition of this functionally essential region by both types of enzymes which act on it. The nature of this RNA structure and its recognition by these enzymes is under study.

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## REFERENCES

1. Fernandez-Puentes, C., and Vazquez, D. (1977) *FEBS Lett.* **78**, 143-146.
2. Hobden, A.N., and Cundliffe, E. (1978) *Biochem. J.* **170**, 57-61.
3. Schindler, D.G., and Davies, J.E. (1977) *Nucleic Acids Res.* **4**, 1097-1110.
4. Endo, Y., and Wool, I.G. (1982) *J. Biol. Chem.* **257**, 9054-9060.
5. Endo, Y., Huber, P.W., and Wool, I.G. (1983) *J. Biol. Chem.* **258**, 2662-2667.
6. Wool, I.G. (1986) In *Structure, Function, and Genetics of Ribosomes* (B. Hardesty and G. Kramer, Eds.), pp. 397-408, Springer-Verlag, New York.
7. Olson, B.H., and Goerner, G.L. (1965) *Appl. Microbiol.* **13**, 314-321.
8. Olson, B.H., Jennings, J.C., Roga, V., Junek, A.J., and Schuurmans, D.M. (1965) *Appl. Microbiol.* **13**, 322-326.
9. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratry Manual*, p. 68, Cold Spring Harbor, NY.
10. Bodley, J.W. (1969) *Biochem.* **8**, 465-475.
11. Amils, R., Matthews, E.A., and Cantor, C.R. (1979) *Methods Enzymol.* **59**, 449-461.
12. Ahn, B.-Y., and Livingston, D.M. (1986) *Mol. Cell. Biol.* **6**, 3685-3693.
13. Hartwell, L.H. (1967) *J. Bacteriol.* **93**, 1662-1670.
14. Hofbauer, R., Fessl, F., Hamilton, B., and Ruis, H. (1982) *Eur. J. Biochem.* **122**, 199-203.
15. Peacock, A.C., and Dingman, C.W. (1967) *Biochem.* **6**, 1818-1827.
16. Berry, M.J., and Samuel, C.E. (1982) *Anal. Biochem.* **124**, 180-184.
17. Hassur, S.M., and Whitlock, H.W. Jr. (1974) *Anal. Biochem.* **59**, 162-164.

18. D'Alessio, J.M. (1985) In *Gel Electrophoresis of Nucleic Acids: A Practical Approach* (D. Rickwood and B.D. Hames, Eds.), pp. 173-197, IRS Press, Oxford.
19. Homann, H.E., and Nierhaus, K.H. (1971) *Eur. J. Biochem.* **20**, 249-257.
20. Hausner, T.-P., Atmadja, J., and Nierhaus, K.H. (1987) *Biochimie* **69**, 911-923.
21. Stirpe, F., Bailey, S., Miller, S.P., and Bodley, J.W. (1988) *Nucleic Acids Res.* **16**, 1349-1357.
22. Sacco, G., Drickamer, K., and Wool, I.G. (1983) *J. Biol. Chem.* **258**, 5811-5818.
23. Endo, Y., Mitsui, K., Motizuki, M., and Tsurugi, K. (1987) *J. Biol. Chem.* **262**, 5908-5912.
24. Endo, Y., and Tsurugi, K. (1987) *J. Biol. Chem.* **262**, 8128-8130.
25. Endo, Y., Tsurugi, K., and Lambert, J.M. (1988) *Biochem. Biophys. Res. Commun.* **150**, 1032-1036.