α-sarcin cleavage of ribosomal RNA is inhibited by the binding of elongation factor G or thiostrepton to the ribosome

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Received October 26, 1990; Revised and Accepted February 22, 1991

ABSTRACT

The translocation reaction catalyzed by elongation factor G (EF-G) is inhibited either by α-sarcin cleavage of 23S rRNA or by the binding of thiostrepton to the *E. coli* ribosome. Here we show that the transitory binding of EF-G and GDP to the ribosome inhibited the rate of α-sarcin cleavage and that stabilization of this binding with fusidic acid completely prevented α-sarcin cleavage. A similar pattern of inhibition was seen upon the binding of elongation factor 2 to the *S. cerevisiae* ribosome. The irreversible binding of the antibiotic thiostrepton to the *E. coli* ribosome, on the other hand, decreased the rate of cleavage by α-sarcin approximately 2-fold. These results suggest that the α-sarcin site is located within the ribosomal domain for EF-G binding and that the conformation of this site is affected by the binding of thiostrepton.

INTRODUCTION

Ribosomal RNA plays a fundamental and direct role in virtually every aspect of ribosome function (1). Many of these roles involve the binding interactions that occur between the ribosome and the ligands (mRNA, tRNA and protein factors) with which it interacts during the course of translation. Studies of the modes of action of ribosomal inhibitors have implicated two specific regions of 23S RNA in the binding of elongation factor G (EF-G) that occurs during the GTP-dependent translocation reaction of protein synthesis elongation. These two regions are the α-sarcin site which comprises a stem and loop involving residues 2646–2674 (2), and the thiostrepton binding site involving residues 1052–1112 (3).

α-Sarcin, a small, basic protein secreted by the mold *Aspergillus giganteus*, inhibits ribosome function by catalytically hydrolyzing a single phosphodiester bond in the 23S-like RNA of the large ribosomal subunit (2). This very specific cleavage occurs in a purine-rich sequence (between residues 2661 and 2662 in *E. coli* 23S RNA and between residues 3025 and 3026 in *S. cerevisiae* 26S RNA) that is highly conserved in the ribosomes of evolutionary diverse organisms. The cleavage event is thought to produce conformational changes in both ribosomal RNA (4,5) and ribosomal proteins (6). This cleavage or its resulting conformational changes, inhibits the ribosomal reactions catalyzed by EF-G (7,8). This same sequence in eukaryotic 23S-like RNA is also acted on by the plant proteins known as the ribosome inactivating proteins (RIPs). Rather than cleaving the phosphodiester backbone of RNA, RIPs remove a single adenine residue (corresponding to residue 2660 in *E. coli* 23S RNA and 3024 in *S. cerevisiae* 26S RNA) by a N-glycolysidic hydrolysis reaction (9–11). This modification has functional consequences similar to those caused by α-sarcin cleavage (8).

Thiostrepton, a modified peptide antibiotic produced by *Streptomyces azureus*, inhibits protein synthesis by binding noncovalently but essentially irreversibly to a specific region of 23S RNA that is distinct from the α-sarcin cleavage site (residues 1052–1112 in *E. coli* 23S RNA; 3). As a result of thiostrepton binding, ribosomes are unable to bind EF-G (12) and conversely, the binding of EF-G prevents subsequent thiostrepton binding (13,14). The mutually exclusive nature of these binding reactions suggests that EF-G and thiostrepton interact at or near the same region of 23S RNA. This region of rRNA is also implicated in the binding of antibiotic, and hence EF-G, by the observation that *S. azureus* produces a specific enzyme that methylates residue 1067 of both *S. azureus* and *E. coli* 23S RNA which renders the methylated ribosomes unable to bind thiostrepton (15).

We report here that the binding of EF-G to *E. coli* ribosomes and elongation factor 2 (EF-2) to *S. cerevisiae* ribosomes prevents α-sarcin cleavage of ribosomal RNA. Furthermore, the binding of thiostrepton to *E. coli* ribosomes reduces the rate of α-sarcin cleavage.

MATERIALS AND METHODS

EF-G and salt-washed 70S ribosomes were prepared from *E. coli* JA300 by methods described before (16). EF-2 and 80S ribosomes were prepared from *S. cerevisiae* SSL204. EF-2, prepared by a modification of the procedure of Skogerson (17),

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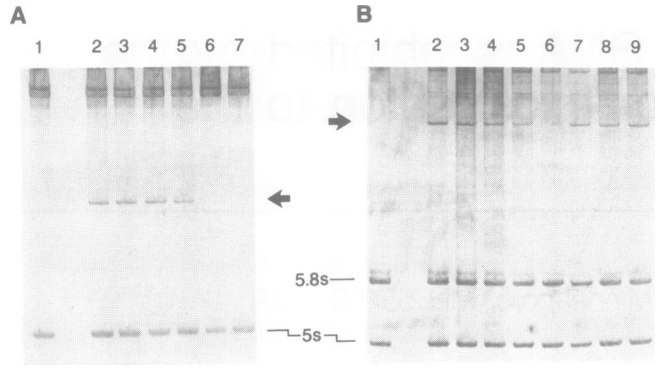

Figure 1. Inhibition of α-sarcin cleavage of ribosomal RNA by the binding to *E. coli* and *S. cerevisiae* ribosomes of GDP, fusidic acid and either EF-G or EF-2. In each case ribosomes were first incubated with varying amounts of factor and then with α-sarcin as described in Materials and Methods. Ribosomal RNA, separated by polyacrylamide gel electrophoresis, was visualized by silver staining following the second incubation. The locations of the α-fragments are designated by the arrows. In each case lane 1 is the control from which α-sarcin was omitted during the second incubation. Panel A, *E coli* ribosomes (20 pmoles) were incubated with varying amounts of EF-G (lanes 1 and 7, 300 pmoles; lane 2, none; lane 3, 0.03 pmoles; lane 4, 0.3 pmoles; lane 5, 3 pmoles; lane 6, 30 pmoles) prior to incubation with α-sarcin. Panel B, *S. cerevisiae* ribosomes (15 pmoles) were incubated with varying amounts of EF-2 (lanes 1 and 6–9, 50 pmoles; lane 2, none; lane 3, 6 pmoles; lane 4, 12 pmoles; lane 5, 25 pmoles) prior to incubation with α-sarcin. Fusidic acid was omitted from the samples analyzed in lanes 7 and 8, and GTP was omitted from the samples analyzed in lanes 7 and 9.

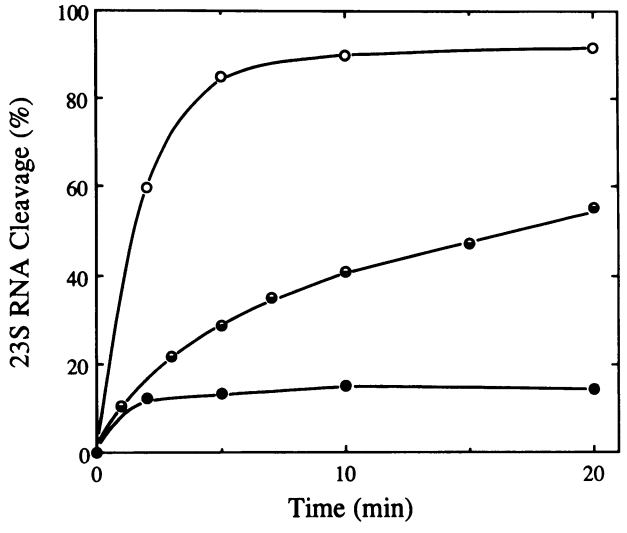

Figure 2. The effect of EF-G binding to *E. coli* ribosomes on the rate of α-sarcin cleavage of 23S RNA. Cleavage with α-sarcin was conducted for varying times after incubating ribosomes (50 pmoles) and EF-G (450 pmoles) with all of the components of the quaternary complex (closed circles) or with all components except fusidic acid (half circles) or EF-G (open circles). The extent of ribosomal RNA cleavage was determined by Northern hybridization of the α-fragment as described in Materials and Methods.

was kindly provided by Dr. M. Donovan. Crude *S. cerevisiae* ribosomes, prepared as described before (18), were salt-washed by centrifugation from a solution containing: 20 mM Tris-acetate (pH 7.5), 1.0 M ammonium acetate, 100 mM $MgCl_2$ and 3 mM 2-mercaptoethanol. *A. giganteus* MDH 18894 was grown in α-sarcin inducing medium and α-sarcin was partially purified from the culture supernatant as previously described (18). The antibiotics fusidic acid and thiostrepton were obtained from Leo Pharmaceutical Products and Squibb, respectively.

The quaternary ribosome·elongation factor·GDP·fusidic acid complex was formed as described earlier (19) with modifications for our present purposes. The complete reaction mixtures in a final volume of 40 μl contained either *E. coli* ribosomes and EF-G or *S. cerevisiae* ribosomes and EF-2 plus 50 lM GTP and 3 mM fusidic acid in Buffer A (20 mM Tris-HCl [pH 7.4], 10 mM NH_4Cl, 10 mM $MgCl_2$, 3 mM 2-mercaptoethanol). The complexes were formed by incubating this solution for 10 min at 0°.

Thiostrepton binding to *E. coli* ribosomes (13) was conducted in a final volume of 25 μl of Buffer A. The antibiotic, dissolved in dimethylsulfoxide, was incubated with 50 pmoles of ribosomes for 10 min at 0°. Control experiments in the absence of thiostrepton contained dimethylsulfoxide at a final concentration of 10% as did those with thiostrepton. When the quaternary complex was formed after thiostrepton binding, the additional components described above were added in a final volume of 15 μl of Buffer A.

Cleavage of rRNA by α-sarcin was conducted for 5 min at 37° with 0.5 lg of the enzyme in a final volume of 50 μl of Buffer A. Reactions were terminated by the addition of an equal volume of 50 mM Tris-HCl (pH 7.4) containing 1% sodium dodecylsulfate. RNA was isolated by phenol extraction and ethanol precipitation. The degree of α-sarcin cleavage of both *E. coli* and *S. cerevisiae* ribosomes was determined qualitatively by staining the 3′-terminal cleavage product (the α-fragment) with silver following electrophoresis on 7.5% polyacrylamide gels. These methods were preformed as described previously (18).

The degree of α-sarcin cleavage of *E. coli* ribosomes was determined quantitatively by Northern hybridization with a probe specific for the α-fragment. RNA prepared as described above and then denatured with glyoxal was separated by electrophoresis on 1.5% agarose gels. The RNA was transferred to a nylon membrane (Nytran, S & S, Inc.), prehybridized and hybridized with a 14-mer probe as suggested by the membrane manufacturer. The 14-mer oligodeoxyribonucleotide probe (CCATTGGCATG-ACA; obtained from National Biosciences, Inc.) was complementary to bases 2696–2709 of *E. coli* 23S RNA. The purified probe was 5′-end labeled using [γ-^{32}P]ATP (New England Nuclear Corp.) and T4 polynucleotide kinase (U.S. Biochemicals) according to the method of Maniatis *et al.* (21). The amount of rRNA cleavage was determined by excising and scintillation counting the bands corresponding to intact 23S RNA and the α-fragment. The results are expressed as percent of total 23S RNA.

RESULTS

The addition of EF-G to the *E. coli* ribosome in the presence of GDP and fusidic acid inhibited the cleavage of 23S RNA by α-sarcin (Fig. 1A). Inhibition of cleavage required an amount of EF-G that is approximately equimolar with ribosomes suggesting that it resulted from the binding of the factor to the ribosome. A similar pattern of inhibition of 26S RNA cleavage was observed upon the addition of EF-2 to *S. cerevisiae* ribosomes (Fig. 1B). The inhibitory effect of EF-2 required the presence of both GDP and fusidic acid (Fig. 1B, lanes 7–9). The requirement for GDP is expected because the binding of factor to the ribosome that occurs in the absence of guanine nucleotides appears to be unrelated to that which occurs during

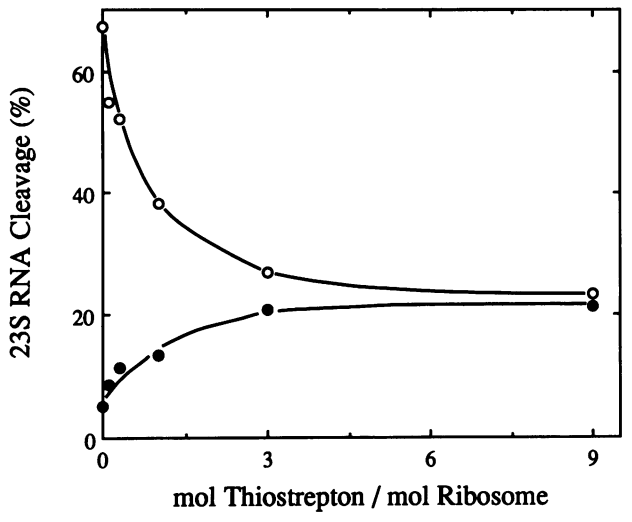

Figure 3. The effect of thiostrepton binding to the *E. coli* ribosome on α-sarcin cleavage of 23S RNA. Thiostrepton, in varying amounts, was first bound to 50 pmoles of ribosomes; then quaternary complex was formed, with (closed circles) and without (open circles) 450 pmoles of EF-G; and finally 23S RNA in ribosomes was cleaved with α-sarcin.

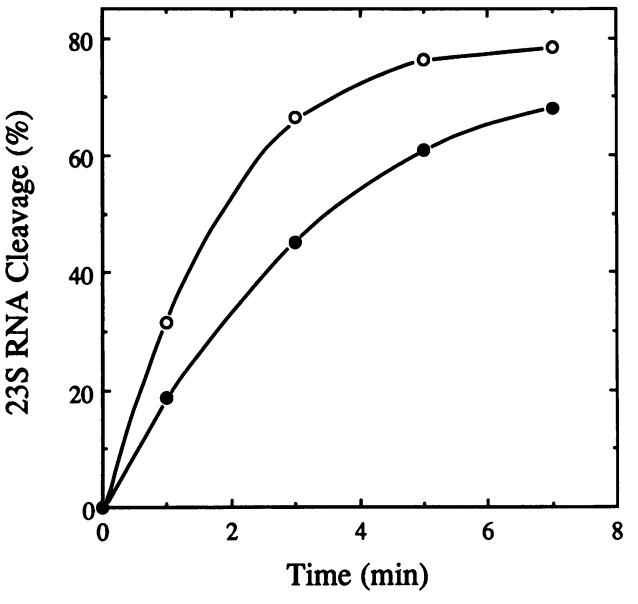

Figure 4. The binding of thiostrepton to ribosomes decreases the rate of 23S RNA cleavage by α-sarcin. Ribosomes (50 pmoles) were incubated with (closed circles) or without (open circles) thiostrepton (1350 pmoles) prior to digestion with α-sarcin.

translocation. The requirement for fusidic acid (Fig. 1B, lane 7) suggests that, under the conditions employed in the experiment shown in Fig. 1B, the transitory binding of EF-2 and GDP in the absence of fusidic acid does not provide protection from α-sarcin cleavage . Thus, the effect on α-sarcin cleavage is in keeping with it resulting from the formation of the quaternary complex of the ribosome, the translocation factor, GDP and fusidic acid. This effect was seen with both prokaryotic and eukaryotic ribosomes and we have utilized *E. coli* ribosomes and EF-G to further analyze its characteristics.

In the absence of other components the extent of RNA cleavage exceeded 90% upon extended incubation of *E. coli* ribosomes with α-sarcin under the conditions shown in Fig. 2 (open circles). When an identical reaction was conducted in the presence of EF-G and GDP, α-sarcin cleavage occurred at a substantially reduced rate (Fig. 2, half circles). When fusidic acid was also present (Fig. 2, closed circles), cleavage corresponding to less than 15% of 23S RNA occurred rapidly but this small degree of cleavage did not increase upon prolonged incubation. These results suggest that α-sarcin is unable to act on ribosomes to which EF-G and GDP are bound. Thus, in the absence of fusidic acid, EF-G and GDP retard the cleavage of rRNA by α-sarcin by binding to and dissociating from the ribosome. In the presence of fusidic acid cleavage does not occur at a detectable rate because EF-G and GDP are unable to dissociate from the ribosome (22).

The binding of thiostrepton to the ribosome also inhibited the cleavage of 23S RNA by α-sarcin (Fig. 3). However, the nature of this inhibition was different from that caused by the binding of EF-G. The open circles in Fig. 3 demonstrate that the maximum inhibition of α-sarcin action is achieved with a quantity of thiostrepton that is approximately equimolar with ribosomes. But under the conditions shown here, the extent of inhibition is not as complete as that observed by the formation of the quaternary complex. The relationship between the effects on α-sarcin cleavage of EF-G and thiostrepton binding is illustrated by the closed circles in Fig. 3. The prior binding of thiostrepton actually *increased* the cleavage of 23S RNA by α-sarcin that occurred in the presence of EF-G, GDP and fusidic acid. This increased cleavage presumably results from thiostrepton inhibition of EF-G binding and from the fact that ribosomes bearing thiostrepton, in contrast to those bearing EF-G, can be acted on by α-sarcin.

The results shown in Fig. 4 demonstrate that thiostrepton binding to ribosomes inhibits the rate of α-sarcin cleavage of 23S RNA by approximately 2-fold. The rate effect of thiostrepton binding is not the result of dissociation of the antibiotic from the ribosome since this binding is known to be essentially irreversible under the conditions employed here (14).

DISCUSSION

The simplest interpretation of the inhibitory effect described here of EF-G on the cleavage of RNA by α-sarcin is that EF-G binding to the ribosome completely occludes the site of α-sarcin interaction. EF-G alone was without effect on the rate of α-sarcin cleavage because the factor is unable to bind to the ribosome in the absence of a guanine nucleotide (22). EF-G in the presence of a guanine nucleotide significantly reduced the rate of α-sarcin cleavage in a manner consistent with a competition between the two proteins for the ribosome. The further addition of fusidic acid completely inhibited cleavage, in accord with the stabilization of factor binding to the ribosome by the antibiotic (22). Garrett and coworkers (23) have also presented evidence that EF-G binding inhibits cleavage by α-sarcin. In addition, RNA footprinting (24) also suggested that EF-G physically interacts with the site of α-sarcin cleavage during its binding to the ribosome. The data reported here extend these findings by showing that EF-G occludes the α-sarcin site. Thus, both functional and structural measurements suggest that the two proteins, EF-G and α-sarcin, interact with an overlapping physical region of the ribosome.

Several observations indicate that EF-G and α-sarcin recognize different aspects of ribosome structure. A small fraction of 23S RNA can be cleaved by α-sarcin in the presence of an excess

of EF-G, GDP and fusidic acid. This likely results from the presence of a fraction of ribosomes that can be acted upon by α-sarcin but is unable to bind EF-G. We have previously shown that a portion of ribosomes, as isolated from *E. coli*, is unable to bind EF-G and that relatively subtle structural changes, including the removal of ribosomal proteins (25), can destroy the ability of ribosomes to bind the factor. In contrast, α-sarcin is capable of specifically cleaving 23S RNA in the complete absence of ribosomal proteins (18). In keeping with these observations, we have found (data not shown) that the specific cleavage of 23S RNA by α-sarcin that occurs in the absence of ribosomal proteins is not affected by the presence of EF-G, GDP and fusidic acid. The modification of RNA by RIPs provides another example of a ribosomal alteration that distinguishes recognition by the translocation factor and α-sarcin. The depurination catalyzed by these proteins inhibits translocation (8) but does not significantly affect the subsequent cleavage of RNA by α-sarcin (11). Clearly, EF-G and α-sarcin interact with the same physical region but do not recognize the same structural features of the ribosome.

Endo and coworkers (26) have described evidence indicating that the degree of α-sarcin cleavage in crude cellular extracts can be altered by the addition of various inhibitors of protein synthesis. On the basis of such observations these authors have suggested that the α-sarcin site may alternate in its structural conformation between an 'open' and 'closed' form during the course of translation and that such a structural alteration might constitute an important aspect of the translocation process. The occlusion of the α-sarcin site by the simple binding of EF-G described here indicates that observations concerning the cleavage of this site in crude extracts containing the factor should be interpreted with caution. Our observations suggest that any circumstance which alters the ribosomal binding of EF-G (or EF-2) in crude extracts would, as a result, also alter the cleavage of ribosomal RNA by α-sarcin.

Our data show that the binding of thiostrepton to the ribosome had a different effect on α-sarcin cleavage, decreasing the rate without significantly altering the extent of cleavage. This decreased rate of cleavage could be the result of a decrease in substrate affinity or in catalytic rate. An alteration in ribosomal conformation upon thiostrepton binding is the most likely cause of this change in the rate with which α-sarcin is capable of cleaving 23S RNA. Thus, the sites of α-sarcin cleavage and thiostrepton binding must communicate with one another.

The results diescribed here, taken together with earlier observations, suggest that the ribosomal site of α-sarcin binding is contained within the ribosomal binding site of EF-G and that the α-sarcin site interacts with but does not overlap the ribosomal binding site of thiostrepton.

ACKNOWLEDGMENT

This work was supported by grant GM-26832 from the NIH.

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