The Catalytic Element of a Ribosomal RNA-processing Complex*

(Bernadette Pace‡, David A. Stahl, and Norman R. Pace‡‡)

From the National Jewish Hospital and Research Center, Department of Molecular and Cellular Biology and the University of Colorado Health Sciences Center, Denver, Colorado 80260

The Bacillus subtilis RNase M5 complex, responsible for the terminal maturation of 5 S rRNA, includes two proteins. One of these proteins is ribosomal protein BL16 (equivalent to Escherichia coli EL18); the other, the α component, is required for catalysis. The RNase M5 α component has been purified in bulk extensively, and the active polypeptide (Mr ≈ 24,000) identified following polyacrylamide gel electrophoresis. Reaction conditions (20–30% dimethyl sulfoxide) are reported which render RNase M5 activity independent of ribosomal protein BL16. This proves that α indeed is the catalytic element, the actual RNase M5, which normally attacks a ribonucleoprotein substrate consisting of protein BL16 in complex with the 5 S rRNA precursor. Kinetic analyses of the BL16-dependent and independent reactions suggest that any α-BL16 association contributes little to the energetics of the α-ribonucleoprotein substrate interaction. It is postulated that the BL16 protein serves as a scaffold, to lock the precursor mRNA into a conformation recognizable by the nuclease.

EXPERIMENTAL PROCEDURES1,2

RESULTS

Vanishingly small quantities of the α protein are present in normal cells, and its overall recoveries initially were poor because the α protein adheres tenaciously to glass and plastic surfaces. Inclusion of a nonionic detergent in all buffers was essential for recovery. We used one of two detergents, NP-40 or Brij 58, at concentrations of 0.01–0.1%. NP-40 has satisfactory solubility properties in all buffers utilized, but absorbs light strongly in the ultraviolet. Brij 58 has no significant UV absorbance, but forms micelles at high ionic strengths in the cold.

We have not yet purified bulk quantities of the α component to homogeneity. We were, however, able to identify the catalytic subunit of the enzyme by recovery in homogeneous state from a denaturing polyacrylamide gel. Fig. 1 shows a silver-stained gel resolving the polypeptides present in an α protein-containing chromatography column peak. The various protein bands were eluted and concentrated from a parallel, Coomassie Blue-stained gel, following the protocol of Hager and Burgess (4), and tested for RNase M5 activity in the standard assay (containing BL16 protein). The activity was associated with the 24,000 molecular weight protein indicated in Fig. 1. Inspection of the eluted α activity on a denaturing gel revealed it to be homogeneous, except for bovine serum albumin added as carrier during manipulations subsequent to electrophoresis (Fig. 1). Moreover, enzyme activity in gradient elution fractions from the carboxymethyl cellulose column clearly paralleled the active polypeptide indicated in the figure (lanes 1–4). Thus, the α component of the RNase M5 is a single polypeptide chain, although it conceivably acts as an oligomer.

The α-RNA Interaction—We have been interested in exploring the nature of the recognition between α, evidently the RNase M5 endonuclease, and its RNA substrate. The fact that the substrate is a ribonucleoprotein complex between the RNA precursor and ribosomal protein BL16 considerably complicates such analysis. However, throughout the partial purification of the α protein, we observed very low capacity to cleave both natural and artificial 5 S RNA precursors in the absence of added BL16 protein. With the notion of reducing the RNase M5 interaction to that of a single RNA and a

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‡ Present address, Department of Biology, Indiana University, Bloomington, IN 47405.

‡‡ Supported by Grant R01 GM 20147 from the National Institutes of Health, United States Department of Health, Education, and Welfare. To whom correspondence should be addressed.

1 Portions of this paper ("Experimental Procedures") are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-0499, cite the authors, and include a check or money order for $1.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: MezSO, dimethyl sulfoxide; NP-40, Nonidet P-40.
The electrophoretic mobility of the single protein, we sought solvent conditions that might promote the α-alone reaction. The inclusion of either high concentrations of Me₂SO in the reaction buffer proved to do so (Fig. 2A). However, the RNase M5 reaction in the presence of Me₂SO was no longer linear with time of incubation; Fig. 2B shows the incremental rates of cleavage (fraction substrate cleaved/min) during the course of the reactions, relative to the first minute of reaction.

course. Apparently the Me₂SO destabilizes some component of the reaction.

The source of the instability of the Me₂SO-dependent reaction was explored to some extent by preincubating the various macromolecular components of the system in reaction buffer containing 25% Me₂SO for 10 min at 37 °C, then adding the remaining components required for the assay. As shown in the autoradiogram of Fig. 3, although the α protein component is to some extent (~50%) inactivated by the preincubation with Me₂SO, the susceptibility of the substrate RNA is almost completely abolished. Gel electrophoresis revealed no degradation of the RNA (data not shown). Presumably, it undergoes refolding in the Me₂SO, adopting a conformation no longer recognized by the nuclease; but we have not explored this phenomenon further.

We were concerned that the Me₂SO might not be alleviating the requirement for the BL16 protein, rather, that it might somehow enhance the activity of some BL16 contaminating even electrophoretically purified α preparations at undetectable levels. The BL16 association with the mature and precursor 5 S RNA is tenuous and turns over rapidly, so the protein potentiates extensive reaction at very low concentrations (2). We therefore raised and purified antiserum against homogeneous BL16 protein ("Experimental Procedures"), and tested for influence of the antiserum on the normal and Me₂SO-promoted RNase M5 reactions. As shown in Fig. 4A, the anti-BL16 serum at high concentrations reduces the α plus BL16 reaction to the α-alone level under standard aqueous conditions. In contrast, the anti-BL16 has no influence on the α-alone reaction in the presence of Me₂SO (Fig. 4B). The possible inactivation of the anti-BL16 serum at the levels of Me₂SO used in the α-alone reactions was tested in a Staphylococcus A protein-binding assay, as shown in Fig. 4C. No significant inhibition of antigen-antibody complex formation was evident. We conclude that Me₂SO alleviates the requirement for protein BL16 in the RNase M5 interaction with the precursor RNA. The essence of the RNase M5 recognition/action process therefore must lie in the α-RNA interaction, with protein BL16 enhancing the reaction in some manner. These experiments also formally prove that the α component indeed is the actual RNase M5 endonuclease.

We compared certain kinetic parameters of the BL16-promoted and Me₂SO-promoted RNase M5 reactions. The Kᵢ values for the substrate were determined for both conditions, and the Kᵢ for mature 5 S RNA was examined in the BL16-dependent reaction, the most biologically relevant. All these determinations were compromised to some extent by the required conditions and the nature of the assay.

Kinetic analyses using these highly radioactive substrates
Catalytic Element of a rRNA-processing Complex

**FIG. 4.** Anti-BL16 serum influence on the BL16-dependent and MeSO4-dependent RNase M5 reactions. A, standard, 10-μl RNase M5 reactions at component concentrations as in Fig. 2, with and without protein BL16 inclusion, were carried out in the presence of 2 μl of the indicated serial dilutions (into 10 mM Tris-HCl, pH 7.6) of anti-BL16 or normal sera (“Experimental Procedures”) and the products analyzed by thin-layer chromatography, as described (14). B, RNase M5 reactions as in A, but containing 25% MeSO4, were carried out in the presence of anti-BL16 serum. C, assay for BL16 antibody function in MeSO4. Standard 3H-staphylococcal A protein-binding assays were carried out as detailed (5), in 0.15 M NaCl, 10 mM sodium phosphate, pH 7.2, with or without 25% MeSO4 as indicated. Microtiter plate wells contained 150 ng (16.7 pmol) of BL16 protein (glutaraldehyde-fixed), 40,000 cpm of 3H-staphylococcal A protein, and 50 μl of antiserum dilution. Following reaction temperature for 1 h, wells were thoroughly rinsed with reaction buffer, then excised for scintillation counting.

are compromised by trace levels of RNA degradation products, generated during preparation and storage, which spill into chromatographic lanes. For accurate product determination, the reactions necessarily are allowed to consume significant amounts of the substrate (>5%). Therefore, measured rates of cleavage do not correspond rigorously to true \( V_0 \) values. Consequently, the reaction sampling times were manipulated to limit substrate consumption to less than 30% of input. The character of the kinetic data (below) suggests that product inhibition had no significant effect on the values obtained.

The MeSO4-promoted reactions were complicated by the apparent instability of the nuclease and the substrate RNA in the presence of 25% MeSO4 (above). The \( K_m \) determinations therefore were evaluated at two early time points during a reaction course. As shown in Fig. 5A, the \( K_m \) values occurred at ~0.15 μM and the \( V_{max} \) at ~10 nmol x liters\(^{-1} \) x min\(^{-1} \), ostensibly at 25 nM α. This corresponds to a turnover number of ~0.4 substrate molecules/min/α monomer polypeptide. Although seemingly low, this is a nominal turnover number, as we have no idea what fraction of polypeptide chains are catalytically active or the number of chains required for a catalytic center.

Kinetic evaluations of the BL16-promoted reactions were done at high concentrations of the BL16, to force the formation of the BL16-RNA complex, the true substrate for the nuclease. The results of the titrations, carried out at 0, 10, and 20 nM mature 5 S RNA, are shown in Fig. 5B. Certain peculiarities of these kinetics and their relevance to the reaction are considered under “Discussion.” Clearly, however, the \( K_m \) for the BL16 complex with the synthetic substrate is ~30 nM and the \( V_{max} \) is ~2.1 nmol x liters\(^{-1} \) x min\(^{-1} \), at 0.625 nM α. This corresponds to a turnover number of ~3.4 substrate molecules/min/α monomer (again, presuming all α molecules present are active as monomers), ~8-fold greater than the MeSO4-promoted reaction. The \( K_m \) for the BL16-mature 5 S rRNA complex is ~10 nM. These values were consistent over many determinations, carried out with a variety of protein and nucleic acid concentrations.

**FIG. 5.** Kinetic analysis of the MeSO4-dependent and BL16-dependent RNase M5 reactions. A, Lineweaver-Burk plot of results from standard reactions containing 25% MeSO4 sampled at 4 and 10 min. Reaction mixtures contained 25 nM α component and varying substrate, as indicated. The abscissa intercept is equal to ~1/\( K_m \); the ordinate intercept, 1/V\(_{max} \) (15). B, standard RNase M5 reactions contained 2.5 nM BL16 protein, 625 pmol α component, varying substrate as indicated. Only part of the data are shown. [S] extended from 0.48 to 2.4 x 10\(^{-6} \) M. Two reaction series, as indicated, contained 10 or 20 nM mature 5 S rRNA, as competitor for the reaction components. In the presence of the competing 5 S RNA, the abscissa intercept in the Lineweaver-Burk plot is 1/K\(_{max} \) (1 + [I]/K\(_m \)) (15). Extrapolated kinetic values are given and discussed in the text.

**DISCUSSION**

Fig. 6 shows the structure of the semisynthetic precursor 5 S rRNA used in these studies, indicating the region of the mature domain which likely interacts with ribosomal protein BL16. The indicated binding site for the reaction-potentiating ribosomal protein is based on experiments examining the protection of Escherichia coli 5 S RNA against chemical and enzyme attack by the E. coli ribosomal protein EL18 (6, 7), evidently the homologue of the B. subtilis BL16 (2). The EL18 protein, in fact, will substitute for BL16 in the RNase M5 reaction.

The RNase M5 reaction is highly specific; no natural or artificial substrates have so far been discovered that do not contain at least part of the mature 5 S RNA. We have shown previously that the overall RNase M5 reaction requires substantial amounts of the mature domain of the precursor RNA (8). That conclusion stems from analyses of the susceptibilities to RNase M5 of test substrates lacking more or less extensive regions of the mature domain. Residues 71–106 (Fig. 6) in the natural precursor RNA could be deleted without abolishing substrate capacity, but a deletion spanning residues ~40–70, a region significantly removed from the site of action, abolished susceptibility to RNase M5. In retrospect, those results likely reflect a requirement for BL16 protein binding, rather than substrate recognition by α, the actual catalyst. Since disruption of a large part of the ribosomal protein-binding site likely would prevent the binding required to promote cleavage by α under normal reaction conditions, the question remains open as to the core substrate requirements.
for recognition by \( \alpha \). We previously observed that a test substrate including only residues 1-14 and 105-116 of the mature domain was not susceptible to cleavage by \( \alpha \) plus BL16 (8). However, this will have to be re-examined using the MeSO-promoted, \( \alpha \)-alone reaction. The information required by \( \alpha \) may be far simpler than we thought, perhaps involving only the short duplex adjacent to the cleavage site (Fig. 6).

Fig. 6 also shows the regions of the 5 S rRNA which, on the basis of protection experiments, associate with the other two ribosomal proteins besides BL16 and EL18 (6, 7). Protein EL25 binds the extended helix comprised of residues 71-100. This region of the 5 S rRNA is not required for the RNase M5 reaction and, indeed, protein EL25 has no influence on the rate of substrate cleavage by the enzyme (2). In contrast, protein EL5 inhibits the BL16-dependent RNase M5 reaction, likely because it associates with the helix containing the RNase M5 cleavage sites and sterically restricts access by the nuclease (2).

The RNase M5 \( \alpha \) component, the specific nuclease, is a relatively simple protein, of monomer molecular weight 24,000 (Fig. 1). This is the size of the RNases III of \( E. \) coli (monomer \( M_r \sim 25,000; \) Ref. 9) and \( B. \) subtilis (monomer \( M_r \sim 27,000; \) Ref. 10). The \( E. \) coli RNase III seems to function as a polypeptide dimer. The \( B. \) subtilis RNase III in solution evidently is a monomer (8), although it conceivably dimerizes during catalysis. We have no information on any oligomeric state of the RNase M5 \( \alpha \) component, although it, too, conceivably functions as a dimeric unit. The reason for considering such is that, in the native substrate, the enzyme must cleave two phosphodiester bonds, arranged with antiparallel orientation in a double helical region (Fig. 6). It seems likely that hydrolysis in the opposed substrate strands could be catalyzed simultaneously by a single catalytic site on the enzyme. This is because the amino acids involved in catalysis likely must consider the polarity of the substrate phosphodiester bonds and these, if inspected simultaneously, would confront the catalytic site with opposite polarities. Thus, the RNase M5 \( \alpha \) component, the nuclease, likely possesses two independent catalytic sites or, alternatively, is constructed as a functional dimer, one catalytic site presented on each identical monomer unit. Nonetheless, highly specific processing nucleases such as RNase M5 or RNase III are not particularly complex proteins, only approximately twice as large in monomer size as "nonspecific" nucleases such as bovine RNase A (\( M_r \sim 14,000; \) Ref. 11) or RNase T1 (\( M_r \sim 11,000; \) Ref. 12).

The fact that the \( \alpha \) component functions without BL16 protein in the presence of MeSO proves that the catalytic specificity lies with \( \alpha \). However, the question arises as to whether BL16 protein contributes significantly to the overall binding energy between \( \alpha \) and the substrate ribonucleoprotein complex, or whether it serves a more passive role. Certainly, the \( \alpha \)-alone reaction is significantly poorer than the BL16-promoted one, with regard to both \( K_a \) and \( V_{\text{max}} \). Some of the reason for the poorer performance of the \( \alpha \)-alone reaction likely is the presence of the MeSO. The solvent inhibits the BL16-promoted reaction, even at modest concentrations, and, at the optimum MeSO concentration for the \( \alpha \)-alone activity, addition of BL16 does not enhance the reaction. We do not know if BL16-RNA substrate complexes form at high concentrations of MeSO; filter binding tests failed under those conditions. Nor do we have a binding assay for \( \alpha \) to the BL16-RNA complex or to free RNA. We, therefore, cannot yet evaluate binding constants and compare, in a straightforward manner, the energetic contribution of BL16 to the substrate interaction with \( \alpha \). However, even the face \( K_a \) values for the MeSO-promoted and the BL16-promoted reactions, which differ by only 25-fold, suggest that BL16 contributes little to the substrate-binding contacts utilized by \( \alpha \). If the \( \alpha \)-substrate \( K_{\text{association}} \) values vary as do the \( K_a \) values, not an unreasonable assumption in these sorts of interactions, then the binding energy between the \( \alpha \) component and the BL16-RNA complex under normal, aqueous conditions would be only \( \sim 15-20 \% \)3 greater than between \( \alpha \) and the naked RNA in the presence of probably inhibitory concentrations of MeSO. Thus, BL16 protein probably contributes few, perhaps no, energetically important contacts between \( \alpha \) and the ribonucleoprotein substrate. However, the BL16 protein certainly is required for the normal reaction, perhaps as a scaffold, converting the mature domain of the precursor into a conformation preferred by \( \alpha \), the catalyst. MeSO is known to destabilize higher-order polynucleotide structure (13). Possibly, the BL16 protein acts similarly, to destabilize the duplex stem adjacent to the site cleaved by the nuclease.

We observed, using substrates lacking various sequences, that the precursor-specific segments have little or no involvement in recognition by \( \alpha \). The important information resides in the mature domain of the precursor RNA (8). The analysis
Catalytic Element of a rRNA-processing Complex

of the inhibition of the maturation reaction by mature 5 S rRNA is consistent with that conclusion. The $K_i$ evaluated with mature 5 S rRNA for the BL16-dependent reaction was $-10$ nM, practically the same as the $K_i$ of the reaction for the BL16-precursor complex (30 nM). However, the inhibition data (Fig. SB) are somewhat irregular; they indicate that the mature 5 S rRNA is a competitive inhibitor (no influence on $V_{max}$), although there is some influence on $K_{eq}$ suggestive of a noncompetitive component, as well. The former is expected if the competition is at the level of $\alpha$ interaction with the RNA-BL16 complex; the latter if the competition were only for BL16 to form active substrate. Probably both considerations pertain in the inhibition analysis, which, because of the high input of mature 5 S rRNA, was carried out at less than saturating BL16 concentrations. The noncompetitive component of the inhibition is slight, however, so the important point is that the $K_i$ value for mature 5 S rRNA (more correctly the mature 5 S RNA-BL16 complex, presumably) is very close to the $K_i$ for the substrate-BL16 complex. Thus, we conclude that $\alpha$ does not discriminate between precursor and mature mRNA. Indeed, it need not do so, as any mature 5 S rRNA in the cell is rapidly incorporated into the ribosome superstructure and hence is removed from the substrate pool. We have not tested whether precursor 5 S rRNA reconstituted into ribosomes is susceptible to cleavage by $\alpha$, but the fact that cleavage is inhibited by the E. coli ribosomal protein EL5 (2), which associates with the 5 S rRNA in the ribosome, predicts that the RNA substrate will not prove to be very accessible to the enzyme there. This suggests that the 5 S rRNA precursors must undergo maturation very early during the course of ribosome assembly.

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REFERENCES


SUPPLEMENTAL MATERIAL TO THE CATALYTIC ELEMENT OF A RIBONUCLEASE PROCESSING COMPLEX BY BERNARDO FACE, DAVID A. STAHL, AND NORMAN R. PACE

EXPERIMENTAL PROCEDURES

Preparation of RNA. E. coli ribosomal protein BL16, gel electrophoresis, and the RNA of the E. coli rRNA are described in the accompanying paper (3). Mg20 was obtained from Fisher Chemical Co. and used without further purification.

Purification of Phase NS 3 Components - 170 g frozen E. coli 16S or 11S rRNA was thawed in 170 ml 7 M KCl buffer (10 mM Tris-HCl, pH 7.1, 750 mM MgCl2). The RNA was then spun at 250,000 × g for 24 h in an SW 50.1 rotor for 15,000 rpm for 18 h in a Beckman SW50.1 rotor. The supernatant was dialyzed overnight against two changes of 7 M KCl buffer. After a film precipitate was formed, the supernatant was loaded onto a 30 ml bed Whatman QAE column equilibrated to 7 M KCl. The supernatant was then washed with QAE buffer to remove the remaining film precipitate. The resulting supernatant was passed through a column of QAE, and then the column was loaded with 0.1 M NaCl. At this point, a sample fractionation was started. A semipreparative detergent buffer (50 mM Tris-HCl, pH 7.5, 40% glycerol) was added to the Whatman QAE column equilibrated to 7 M KCl buffer, then washed with the same buffer. Two peaks of activity were separated and used for the last steps of purification. The activity was pooled, and the column fractions were pooled. A semipreparative detergent buffer (50 mM Tris-HCl, pH 7.5, 40% glycerol) was added to the Whatman QAE column equilibrated to 7 M KCl. The resulting supernatant was loaded onto a column of QAE and then loaded onto a column of a QAE column equilibrated to 7 M KCl. After the final column, the column fractions were analyzed and the active fractions were pooled. The final step of purification was the use of a native detergent buffer (50 mM Tris-HCl, pH 7.5, 40% glycerol) and the final step of purification was the use of a native detergent buffer (50 mM Tris-HCl, pH 7.5, 40% glycerol). After a film precipitate was formed, the supernatant was dialyzed overnight against two changes of 7 M KCl buffer. After a film precipitate was formed, the supernatant was loaded onto a column of QAE, and then the column was loaded with 0.1 M NaCl. At this point, a sample fractionation was started. A semipreparative detergent buffer (50 mM Tris-HCl, pH 7.5, 40% glycerol) was added to the Whatman QAE column equilibrated to 7 M KCl. The resulting supernatant was loaded onto a column of QAE and then loaded onto a column of a QAE column equilibrated to 7 M KCl. After the final column, the column fractions were analyzed and the active fractions were pooled. The final step of purification was the use of a native detergent buffer (50 mM Tris-HCl, pH 7.5, 40% glycerol) and the final step of purification was the use of a native detergent buffer (50 mM Tris-HCl, pH 7.5, 40% glycerol).