Purification and Characterization of the tRNA-processing Enzyme RNase BN*

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RNase BN, a tRNA-processing enzyme previously shown to be required for the 3'-maturation of certain bacteriophage T4-encoded tRNAs, was overexpressed and purified to near homogeneity from *Escherichia coli*. The purified enzyme, which is free of nucleic acid, is an α2-dimer with a molecular mass of ~65 kDa. RNase BN displays a number of unusual catalytic properties compared with the other exoribonucleases of *E. coli*. The enzyme is most active at pH 6.5 in the presence of Co²⁺ and high concentrations of monovalent salts. It is highly specific for tRNA substrates containing an incorrect residue within the universal 3’-CCA sequence. Thus, tRNA-CU and tRNA-CA are effective substrates, whereas intact tRNA-CCA, elongated tRNA-CCA-Cn, phosphodiesterase-treated tRNA, and the closely related tRNA-CC are essentially inactive as substrates. RNA or DNA oligonucleotides also are not substrates. These data indicate that RNase BN has an extremely narrow substrate specificity. However, since tRNA molecules with incorrect residues within the -CCA sequence are not normally produced in *E. coli*, the role of RNase BN in uninfected cells remains to be determined.

Thus, these strains do not support the growth of a mutant T4 phage (BU33) because a phage-encoded suppressor tRNA*<sup>sec</sup>, required for the translation of an amber mutation in a BU33 head protein, cannot be processed to its mature form due to a defect in 3’-maturation (9). The molecular basis for this phenotype in strains BN and CAN is the deficiency of the exoribonuclease RNase BN (10, 11). Thus, in contrast to uninfected cells, in which at least five exoribonucleases can remove the extra residues following the -CCA sequence during 3’-maturation of tRNA precursors, only RNase BN appears able to remove incorrect residues within the -CCA sequence during phage T4 tRNA maturation.

To learn more about the structure, function, and specificity of RNase BN, we first identified and cloned the *rbn* gene encoding the enzyme (12). We discovered that *rbn* is nonessential in *E. coli* and that it encodes a polypeptide of 32.8 kDa (12). In this paper, we describe the overexpression, purification, and characterization of RNase BN.

EXPERIMENTAL PROCEDURES

**Strains and Plasmids—**Plasmid pBS*<sup>rbn</sup>* (12) was used to overexpress RNase BN. *E. coli* strain BL21(DE3) pLys (13) was used as the host for the aforementioned plasmid.

**Culture Conditions—**Cells were routinely grown at 37 °C in YT medium or on YT plates (14). Ampicillin, when added, was present at a concentration of 200 or 300 μg/ml. Growth in liquid medium was followed by absorbance measurements at 600 nm.

**Materials—**[³²P]ATP and [³H]Poly(A), blue dextran 2000, Ultrogel AcA44, and DEAE-Sephadex A-50 were obtained from Amersham Pharmacia Biotech. [³H]ATP and [³²P]ATP were obtained from NEN Life Science Products. Unlabeled poly(A) used to dilute the radioactive material was obtained from Sigma. Bacterial alkaline phosphatase was purchased from Worthington. N-Ethylmaleimide, 5,5'-dithiobis(2-nitrobenzoic acid), p-hydroxymercuribenzoate, and protein size markers were obtained from Sigma. Mercuric chloride was obtained from J. T. Baker Inc. Hydroxylapatite HT, Affi-Gel blue, and cellulose were purchased from Bio-Rad. All other chemicals were reagent-grade.

**RNase BN Assay—**RNase BN removes the 3'-terminal mononucleotide AMP from the phage tRNA precursor analogue tRNA-C<sup>[¹⁴C]</sup>A. This substrate was prepared as described previously (15). Reaction mixtures of 100 μl contained 20 mM Hepes, pH 6.5, 0.2 mM CoCl<sub>2</sub>, 200 mM KCl, 8 or 16 μg of tRNA-C<sup>[¹⁴C]</sup>A (10<sup>6</sup> cpm/nmol), and cell extract or purified enzyme. Samples were incubated for 30 min at 37 °C, and acid-soluble radioactivity was determined. One unit of RNase BN is the amount of enzyme that releases 1 nmol of AMP in 1 h.

**Overexpression of RNase BN—**Plasmid pBS*<sup>rbn</sup>* carrying the *rbn* gene cloned under control of a T<sub>7</sub> promoter was transformed into strain BL21(DE3) pLys. Bacteriophage DE3 contains the gene for T<sub>7</sub> RNA polymerase controlled by the lacUV5 promoter, which is inducible by isopropyl-β-D-thiogalactopyranoside. The pLys plasmid carries the gene for T<sub>7</sub> lysozyme, which inhibits basal levels of T<sub>7</sub> transcription in the uninduced cell. Cells were grown to an absorbance of 0.5 at 600 nm in YT medium containing 200 μg/ml ampicillin. To maintain the *rbn*<sup>+</sup> plasmid, cells were then harvested by centrifugation at 5000 rpm for 5 min, washed with YT medium to remove β-lactamase, and then resuspended in fresh YT medium containing 300 μg/ml ampicillin. The cul-

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Purification and Characterization of RNase BN

TABLE I
Summary of purification of RNase BN

| Step                    | Total Protein | Total activity | Specific activity | Relative purification | Recovery |
|-------------------------|---------------|----------------|-------------------|-----------------------|----------|
|                         | mg            | units          | units/mg          | -fold                 | %        |
| 1. High-speed supernatant | 59            | 1491           | 25.7              | 1.0                   | 100      |
| 2. DEAE-Sephadex A-50   | 43.5          | 3588           | 82.4              | 3.2                   | 240      |
| 3. Hydroxylapatite      | 19.8          | 3300           | 167               | 6.5                   | 221      |
| 4. Ultrogel-AAc44       | 4.9           | 1277           | 261               | 10                    | 85       |
| 5. Affi-Gel blue*       | 0.6           | 732            | 1200              | 46                    | 49       |

* Based on the sum of the peak and side fractions.

In a 25-fold overproduction of RNase BN during the 4-h induction period. In several experiments, it was found that this amount of time gave maximum expression of RNase BN.

As shown in Table I, RNase BN was purified from a high-speed supernatant fraction using a series of four chromatographic steps. The overall purification from the overexpressed extract was 46-fold, equivalent to ~1000-fold from a normal extract. The apparent recovery was 50%; however, as is clear from the increased activity in steps 2 and 3, RNase BN activity in the supernatant fraction actually is inhibited 2–3-fold, presumably by the large amount of nucleic acid present. This inhibition was verified directly by mixing of purified RNase BN with the initial supernatant fraction (data not shown).

Purified RNase BN was stored for 1 month under a variety of conditions and then re-assayed to determine optimal conditions for storage. Although the enzyme was relatively stable under all the conditions tested, it was most stable at −20 °C in the presence of 50% glycerol or at −80 °C after rapid freezing in a dry ice/ethanol bath. Under these conditions essentially no RNase BN activity was lost during the period of storage.

Purity of RNase BN—Based on the close correspondence between RNase BN activity and absorbance at 280 nm upon elution from Affi-Gel blue (Fig. 1), it was apparent that RNase BN was quite pure. This was confirmed by SDS-polyacrylamide gel electrophoresis (Fig. 2). Overexpression of RNase BN is evident by comparison of lanes 6 and 7. The progressive purification of RNase BN can be seen in lanes 6 to 2. We estimate that the most purified material (lane 2) was at least 95% pure.

Spectral analysis of RNase BN, carried out in the range of 200–350 nm, revealed no unusual peaks (data not shown). The A_{280}/A_{260} ratio was 1.89, indicating that purified RNase BN is free of nucleic acid. Thus, RNase BN does not require nucleic acid for activity.

Molecular Mass and Subunit Structure—SDS-polyacrylamide gel electrophoresis (Fig. 2) indicated that purified RNase BN contains only a single species of polypeptide chain with a molecular mass of ~37 kDa. This is in good agreement with the predicted size of RNase BN of 32.8 kDa based on the sequence of the rbn gene (12).

Based on gel filtration on Ultrogel-AAc44 (step 4 of the purification procedure) and assuming that it is a globular protein, the native molecular mass of RNase BN is ~65 kDa. These data strongly support the conclusion that RNase BN functions as an α_{2}-dimer, as previously suggested from earlier genetic studies in which an interruption-deletion mutant of RNase BN displayed a dominant-negative effect (12).

Effect of Sulphydryl Reagents—Based on the nucleotide sequence of the rbn gene, RNase BN contains 2 cysteine residues/polyepitope chain (12). To ascertain whether these residues are present in their reduced form and whether they might play a role in enzyme activity, purified RNase BN was incubated with the sulphydryl reagents N-ethylmaleimide, 5,5'-dithiobis(2-nitrobenzoic acid), p-hydroxymercuribenzoate, and mercuoric chloride. As presented in Table II, each of these reagents was

1. C. Callahan and M. P. Deutscher, unpublished observation.
found to be a potent inhibitor of RNase BN, decreasing RNase BN activity ~90% upon preincubation for 30 min at 37 °C. These data show that at least 2 of the cysteine residues in native RNase BN are in the reduced form and that cysteine residues are required for enzyme activity. Whether these residues are directly required for catalysis or indirectly by maintaining structure, such as the dimeric form, remains to be determined.

**Temperature Sensitivity of RNase BN**—Samples of RNase BN were incubated at various temperatures for 10 min prior to determining the amount of activity remaining. The data in Fig. 3 show that RNase BN remained stable for 10 min at temperatures up to 45 °C, and at 50 °C, still retained 66% of its activity. However, at 60 °C, 90% of the activity was lost in 10 min. Additional experiments revealed that RNase BN retained ~25% of its activity upon incubation for 40 min at 50 °C (data not shown). These data indicate that RNase BN is moderately stable at elevated temperatures.

**Optimal Conditions for RNase BN Activity**—RNase BN was assayed under a variety of conditions to assess the requirements for optimal activity. Assay at pH values between 6.0 and 8.0 in Mes and Hepes buffers indicated that RNase BN is most active at pH 6.5. Activity at pH 8.0 was 70% of that at pH 6.5, whereas that at pH 6.0 was 95% of that at pH 6.5. The relatively low pH optimum for RNase BN is unusual among the exoribonucleases of *E. coli*, which generally function optimally at more alkaline pH values.

Among the divalent cations tested, significant activity was observed only with Co²⁺ and Mg²⁺, with Co²⁺ being considerably more effective at 0.2 mM (Table III). A more detailed examination of these two cations (Fig. 4) revealed that at its optimum (0.2 mM), Co²⁺ was approximately twice as effective as Mg²⁺ at its optimum (1 mM). Both cations displayed quite sharp optima. These findings are quite unusual as Co²⁺ has not been observed previously to function as such an effective cofactor for RNases.

RNase BN also requires a monovalent cation for activity. With K⁺ as the cation, the optimal concentration was 200–400 mM (Fig. 5). At 200 mM, the monovalent cation requirement could be satisfied by Li⁺, which was as effective as K⁺; Na⁺ and Rb⁺ were ~70% as effective, and Cs⁺ was ~30% as effective. A monovalent cation is also required when Mg²⁺ is the divalent cation. Although a number of other *E. coli* exoribonucleases are stimulated by monovalent cations, the absolute requirement of RNase BN for a monovalent cation is unusual.

**Substrate Specificity of RNase BN**—RNase BN is highly spe-

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*The abbreviation used is: Mes, 4-morpholineethanesulfonic acid.*
related tRNA-CC, which differs from the active substrates by a single terminal residue, was almost inactive. Given this high degree of specificity against such similar molecules, it is not surprising that rRNA and poly(A) also were poor substrates (Table IV).

A second group of substrates consisting of RNA and DNA oligonucleotides 11–17 residues in length were also tested as substrates (Table V). The sequences of the RNA oligonucleotides are those of the 3' terminus of E. coli tRNA\(^{[\text{[14C]}]}\) and its precursor molecules with 3 or 6 extra 3'-residues. These molecules were labeled at their 5' termini with \(^{32}\text{P}\), and RNase BN action was assessed on a 20% acrylamide gel, which can detect shortening by even a single nucleotide residue. Based on this analysis, none of these molecules were substrates for RNase BN under standard assay conditions.

Products of RNase BN Action—Based on earlier work in which relatively crude preparations of RNase BN were found to release UMP from tRNA-CU, it was suggested that this enzyme is an exoribonuclease (10). To confirm this conclusion and to ensure that the release of mononucleotide was not due to a secondary reaction resulting from the presence of a contaminating activity, the products of the reaction catalyzed by highly purified RNase BN were examined. Thus, using tRNA-C\(^{[14C]}\)A as a substrate, both the acid-soluble and tRNA products were determined (Table VI).

If RNase BN were an exoribonuclease, the \(^{14}\text{C}\)-labeled acid-soluble product would be expected to be AMP, which, upon treatment with alkaline phosphatase, would be converted to the uncharged molecule \(^{14}\text{C}\)adenosine. If, on the other hand, RNase BN were an endoribonuclease, any acid-soluble oligonucleotides produced would remain charged after treatment with the phosphatase and would elute with the "nucleotide" fraction. Columns of the anion exchanger Dowex AG 1-X2 were used to separate radioactive nucleoside from nucleotide species. As shown in Table VI, after phosphatase treatment, 93% of the acid-soluble radioactivity was eluted from the ion-exchange column with water, consistent with its being the nucleoside adenosine.

To further verify the exoribonucleolytic action of RNase BN, the tRNA product was treated with tRNA nucleotidyltransferase in the presence of \(^{3}\text{H}\)CTP. Removal of AMP from tRNA-C would generate tRNA-C, a substrate for the incorporation of CMP by tRNA nucleotidyltransferase. As also shown in Table VI, release of 14 pmol of acid-soluble radioactive tRNA-C\(^{[14C]}\)A by RNase BN resulted in the incorporation of 13 pmol of \(^{3}\text{H}\)CTP over and above that incorporated in the absence of RNase BN action. The incorporation of \(^{3}\text{H}\)CTP in the absence of RNase BN treatment is due to the fact that the preparation of the tRNA-C\(^{[14C]}\)A substrate from tRNA-C is not quantitative, and some residual RNA-C remains. Nevertheless, these data support the conclusion that RNase BN removed

**TABLE III**

| Substrate | RNase BN activity (pmol/h) |
|-----------|---------------------------|
| None      | 4                         |
| Co\(^{2+}\) | 29                       |
| Mn\(^{2+}\) | 12                       |
| Cd\(^{2+}\) | 6                        |
| Ca\(^{2+}\) | 1                        |
| EDTA      | 3                        |

**FIG. 4.** Effect of Co\(^{2+}\) or Mg\(^{2+}\) concentration on RNase BN activity. Purified RNase BN (0.05 μg) was assayed with 16 μg of tRNA-C\(^{[14C]}\)A and the indicated concentrations of divalent cation as described under "Experimental Procedures."

**FIG. 5.** Effect of KCl concentration on RNase BN activity. Purified RNase BN (0.025 μg) was assayed with 8 μg of tRNA-C\(^{[14C]}\)A and the indicated concentrations of KCl as described under "Experimental Procedures."

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**TABLE IV**

| Substrate | RNase BN activity (pmol/h) |
|-----------|---------------------------|
| [\(^{32}\text{P}\)]tRNA | <5               |
| [\(^{32}\text{P}\)]Diesterase-treated tRNA | <5               |
| [\(^{3}\text{H}\)]tRNA | <5               |
| tRNA-CC\(^{[14C]}\)A | 5                |
| tRNA-A\(^{[14C]}\)C | 68              |
| tRNA-A\(^{[14C]}\)U | 133             |
| tRNA-CCA\(^{[14C]}\)Cn | <5             |
| [\(^{3}\text{H}\)]Poly(A) | 5                |
The significance of their multimeric forms will have to await further structural information about this class of enzymes, so the sig-
tifications with substrate. As yet, there is very little detailed reports on the interac-
monality in their quaternary structure. Perhaps, this repre-
shown are the averages of two experiments.
remains charged after phosphatase treatment.
Nucleosides were eluted with seven 1-ml portions of H2O, and nucleo-
products of RNase BN treatment of tRNA-C[14C]A
\begin{table}[h]
\centering
\caption{Purification and Characterization of RNase BN}
\begin{tabular}{|c|c|c|}
\hline
Product of RNase BN treatment of tRNA-C[14C]A & Amount of product & Product due to RNase BN & After treatment with phosphatase with \begin{tabular}{c}
\hline
Nucleoside \text{ pmol}
\end{tabular}
\begin{tabular}{c}
\hline
Nucleotide \% \text{ pmol}
\end{tabular}
\begin{tabular}{c}
\hline
\end{tabular}
\hline
Acid-soluble & 14 & 14 & 93 & 7 & \text{pmol} & \text{pmol} & \begin{tabular}{c}
\%
\end{tabular}
\hline
[3H]CTP into tRNA & 32 & 13 & 19 & & & &
\hline
(+RNase BN) & & & & & & &
\hline
[3H]CTP into tRNA & & & & & & &
\hline
(-RNase BN) & & & & & & &
\hline
\end{table}

The nucleotide fraction would contain any acid-soluble species that remains charged after phosphatase treatment.
a single nucleotide residue from tRNA-C[14C]A to generate [3H]AMP and tRNA-C, as expected if it were an exoribonuclease.

**DISCUSSION**

In this paper, the purification of RNase BN in its native untagged form has been described. RNase BN is now the sev-
ity of the eight known E. coli exoribonucleases (all except RNase R) to have been purified to near homogeneity. Interest-
ly, RNase BN is the sixth of the eight enzymes to exist as a multimer and the fifth to be an \( \alpha_2 \)-dimer (polynucleotide phospholysis is a trimer). Inasmuch as there is relatively little overall sequence similarity or size in among the exoribonucleases as a group, it is intriguing that there is com-
monality in their quaternary structure. Perhaps, this repre-
sents some consistency among these enzymes in their interactions with substrate. As yet, there is very little detailed structural information about this class of enzymes, so the sig-
nificance of their multimeric forms will have to await further work.

In contrast to its shared structural similarity, RNase BN is unique among the E. coli exoribonucleases in a number of its catalytic properties. These include a low pH optimum of 6.5, a strong preference for \( \text{Co}^{2+} \) as the required divalent cation, and a requirement for a high concentration of monovalent ions or for elevated ionic strength. In addition, RNase BN displays a highly unusual and strict substrate specificity. Of the mole-
cules tested, only those containing alterations within the 3’-
ternal -CCA sequence serve as effective substrates, resulting in removal of the incorrect nucleotide. Other closely related molecules, even tRNA-CC, are inactive or very poorly active as substrates. This unusual substrate specificity makes RNase BN ideally suited for its, so far, only known biological role: the maturation of a subset of bacteriophage T4 tRNA precursors that lack the -CCA sequence (5). RNase BN is essential for the 3’-processing of at least some of this group of tRNAs (6, 9).

This, of course, raises the interesting question of what the role of RNase BN is in the uninfected E. coli cell. Clearly, this enzyme has not been maintained solely for its role during phage infection. It is known that RNase BN can contribute to the maturation of cellular tRNAs when other processing exoribo-
nucleases are absent (3, 4). However, it is the poorest of all the exoribonucleases in this regard. RNase BN is not an essential enzyme in E. coli, and its absence has no effect on cell growth under usual laboratory conditions (12). It is possible that RNase BN is important during a stress response. In fact, RNase BN expression is affected by manipulation of certain proteins altered during heat shock, but further work is necessary to clarify this effect. One might expect that the unusual substrate specificity of RNase BN would be a clue to its cellular function. Yet in E. coli, in contrast to higher organisms, all tRNA genes encode the universal -CCA sequence (17). Thus, there are no known conditions in which incorrect residues would be present in this sequence other than in the case of errors during transcription.

From the foregoing discussion, it is evident that there is much remaining to be learned about the structure and function of RNase BN. Now, with the availability of purified protein and of mutant strains lacking RNase BN (12), continued progress can be expected in studies of this interesting enzyme.

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