Characterization of Escherichia coli RNase PH*

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We have previously shown that the orfE gene of Escherichia coli encodes RNase PH. Here we show that the OrfE protein (purified as described in the accompanying paper) (Jensen, K. F., Andersen, J. T., and Poulsen, P. (1992) J. Biol. Chem. 267, 17147–17152) has both the degradative and synthetic activities of RNase PH. This highly purified protein was used to characterize the enzymatic and structural properties of RNase PH. The enzyme requires a divalent cation and phosphate for activity, the latter property indicating that RNase PH is exclusively a phosphorolytic enzyme. Among tRNA-type substrates, the enzyme is most active against synthetic tRNA precursors containing extra residues following the -CCA sequence, and it can act on these molecules to generate mature tRNA with amino acid acceptor activity; 3'-phosphoryl-terminated molecules are not active as substrates. The equilibrium constant for RNase PH is near unity, suggesting that at the phosphate concentration present in vivo, the enzyme would participate in RNA degradation. The synthetic reaction of RNase PH displays a nonlinear response to increasing enzyme concentrations, and this may be due to self-aggregation of the protein. Higher order multimers of RNase PH could be detected by gel filtration at higher protein concentrations and by protein cross-linking. The possible role of RNase PH in tRNA processing is discussed.

RNase PH is one of seven exoribonucleases known to be present in Escherichia coli (1). The enzyme was first identified by its phosphorolytic activity against natural precursor tRNAs and tRNA analogues from which it removes residues downstream of the -CCA sequence (2, 3). Like the other phosphorolytic exoribonuclease, polynucleotide phosphorylase, RNase PH is also able to carry out a synthetic reaction in which nucleoside diphosphates are used to add residues onto the 3' terminus of RNA molecules (4, 5). Although the primary function of RNase PH in vivo has not been elucidated, the enzyme has been found to be essential for E. coli viability in cells already lacking the RNases and phage T7. Moreover, RNase PH affects tRNA biosynthesis in cells devoid of certain of the exoribonucleases.2

RNase PH was previously partially purified by chromatography, and subsequently identified as a distinct band on SDS-PAGE (6). N-terminal sequence analysis of the first 21 amino acids from this protein band revealed that RNase PH corresponded to the product of the orfE gene (6), a previously unidentified open reading frame upstream of, and co-transcribed with, pyrE (7). Jensen’s (8) laboratory has recently developed an overproduction system in which milligram quantities of the OrfE protein can be obtained with only two purification steps; this work is reported in the accompanying paper. The availability of highly purified OrfE protein presented us the opportunity to assess the protein’s catalytic properties.

In this paper we show, first of all, that the OrfE protein possesses both the degradative and synthetic activities of RNase PH. We then used the highly purified protein to perform a detailed characterization of RNase PH.

EXPERIMENTAL PROCEDURES

Materials—[3H]Poly(A) and [3H]amino acids were obtained from Amersham and [3H]CDP and [3H]CTP were obtained from Du Pont-New England Nuclear. Poly(A), used to dilute the radioactive material, was obtained from Sigma. Ovalbumin, bacterial alkaline phosphatase, and blue dextran 2000 were purchased from Sigma, Cooper Biomedical, and Pharmacia Inc., respectively. N-Ethylmaleimide (NEM) and p-hydroxymercurobenzoate (PMB) were obtained from Sigma. Dimethyl suberimidate (DMS) was from Pierce Chemical Co. Ultragel AcA 44 was purchased from Pharmacia LKB Biotechnology Inc. All other chemicals were reagent grade.

The tRNA substrates for the RNase PH degradative reaction, tRNA-CCA-[3H]Cp, tRNA-CCA-[14C]Cp, and tRNA-CCA-[3H]Cp were prepared as described previously (9). tRNA-CCA-[3H]Cp was prepared from tRNA-CCA-[3H]Cp2 by periodate oxidation using a modification of the procedure of Evans and Deutscher (10). Forty µg of tRNA-CCA-[3H]Cp2 were incubated for 30 min at 45 °C in 0.2 M lysine, pH 8.8, in the presence or absence of 30 nmol of sodium periodate in a volume of 20 µl. The tubes were placed in ice for 15 min and excess periodate was destroyed with 2 µl of 10-4 M ethylene glycol by incubation at room temperature for 20 min. These RNAs were used directly as substrates for RNase PH or were first treated with bacterial alkaline phosphatase (0.46 µg for 30 min at 45 °C) to remove any 3'-terminal phosphate produced.

Partially purified RNase PH was prepared as described earlier (5). Highly purified OrfE/RNase PH was kindly provided by Dr. Kaj Frank Jensen, University of Copenhagen, and its purification is described in the accompanying paper (8).

RNase PH Degradative Assay—This assay measured the phosphate-dependent release of radioactivity from tRNA-CCA-[3H]Cp2. Reaction mixtures of 100 µl contained: 50 mM Tris-Cl, pH 8.0, 50 mM KC1, 5 mM MgCl2, ±15 mM NaPO4, and 10 µg of RNA substrate. Usually, 0.03-0.04 µg of purified enzyme was added and samples were incubated for 10 or 30 min at 37 °C. Trichloroacetic acid-soluble radioactivity was determined as described (3). In all cases activity in the absence of Pi was subtracted so that only Pi-dependent activity is reported.

RNase PH Synthetic Assay—This assay measures the incorpora-

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3 The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; tRNA-CCA-[3H]Cp2, mature tRNA to which an average of 2 to 3 [3H]CMP residues were added; NEM, N-ethylmaleimide; PMB, p-hydroxymercurobenzoate; DMS, dimethyl suberimidate.
tion of [\(^{3}H\)]CDP into tRNA as acid-precipitable radioactivity (6). Reaction mixtures for the synthetic reaction contain in 100 µl: 50 mM glycine-NaOH, pH 9.75, 5 mM MgCl\(_2\), 75 mM KCl, 0.5 mM [\(^{3}H\)]CDP, 17 µg of tRNA, and the indicated amount of RNase PH. Incubation conditions are presented in the legends. Acid-cotica-preparatoactivity, as described above.

**Results**

Comparison of OrfE Protein and RNase PH—To ensure that the OrfE protein was appropriate for the characterization of RNase PH, we have compared the highly purified protein to partially purified RNase PH prepared in our laboratory on the basis of its RNase PH activity. Both proteins were found to migrate identically on 12.5% SDS-PAGE (12) and then silver stained.

**Cross-linking of RNase PH**—Varying amounts of RNase PH were added to 0.25 M tris(ethanolamine, pH 8.4, 10% glycerol, 0.05 mM phenylmethylsulfonyl fluoride, 0.05 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.05 mM dithiothreitol, 0.05 mM EDTA, and then loaded onto the column; the ovalbumin was found to stabilize RNase PH upon dilution on the column. Fractions (0.26 ml) were collected and assayed for both the synthetic and degradative activities. The column was standardized with ovalbumin, bacterial alkaline phosphatase, and blue dextran 2000.

The standard degradative reaction was carried out with the indicated divalent cations. Approximately 0.04 µg of purified RNase PH was assayed for 10 min at 37°C.

### Table I

| Condition  | RNase PH degradative activity (pmol) | 5 mM Mg\(^{2+}\) | 1 mM EDTA | 1 mM Mn\(^{2+}\) | 5 mM Mn\(^{2+}\) | 1 mM Co\(^{2+}\) | 5 mM Co\(^{2+}\) |
|------------|--------------------------------------|-----------------|-----------|-----------------|-----------------|---------------|---------------|
| Complete reaction | 100 | 71 | 43 | 29 | 21 | 20 | 13 |
| -KCl | 54 | 30 |
| -NaPO\(_4\) | -5 |

8–9. The optimal Mg\(^{2+}\) concentration was 5–10 mM. KCl stimulated RNase PH activity up to 2-fold at 50–75 mM. Above this concentration KCl became inhibitory. Maximum RNase PH activity was obtained at 10 mM NaPO\(_4\), with relatively little change in activity up to at least 40 mM.

Other divalent cations were tested to see if they could substitute for Mg\(^{2+}\) in the degradative reaction (Table II). Mn\(^{2+}\), Co\(^{2+}\), and Zn\(^{2+}\) were tested at concentrations of 1 and 5 mM. Both Mn\(^{2+}\) and Co\(^{2+}\) were found to substitute for Mg\(^{2+}\) to some degree (about 40 and 30%, respectively). In both cases 1 mM cation was somewhat more effective than 5 mM, in contrast to the situation with Mg\(^{2+}\). Essentially no activity was observed when Zn\(^{2+}\) was substituted for Mg\(^{2+}\).

Effect of Enzyme Concentration on the Synthetic and Degradative Reactions—Previously, RNase PH, in a partially purified fraction, was shown to respond linearly to enzyme concentration in the degradative reaction, but to give a distinctly nonlinear response in the synthetic reaction (5). To determine whether this unusual situation might have been dependent on other proteins in the crude preparation, we have re-examined this phenomenon with the purified enzyme. The data in Fig. 1 demonstrate that the same result is found with the highly purified protein; i.e. the degradative reaction is linear with respect to the amount of enzyme added, whereas the synthetic reaction is greatly stimulated at higher amounts of RNase PH. These data show that the unusual response of the synthetic reaction to increasing enzyme is an intrinsic property of RNase PH.

### Table II

| Divalent cation | RNase PH degradative activity (pmol) |
|----------------|--------------------------------------|
| 5 mM Mg\(^{2+}\) | 71 |
| 1 mM EDTA | 43 |
| 1 mM Mn\(^{2+}\) | 29 |
| 5 mM Mn\(^{2+}\) | 21 |
| 1 mM Co\(^{2+}\) | 20 |
| 5 mM Co\(^{2+}\) | 13 |
| 1 mM Zn\(^{2+}\) | <3 |
| 5 mM Zn\(^{2+}\) | <3 |
Enzyme was added to the standard degradative reaction mixtures and to degrade poly(A) and the ratio relative to tRNA-CCA-polynucleotide phosphorylase and RNase PH, respectively. PH against the nonspecific substrate, poly(A), was an intrinsic property of the enzyme or might have been due to a contaminating RNase in the crude preparation. As also shown in Table III, highly purified RNase PH retains its ability to degrade poly(A) and the ratio relative to tRNA-CCA-[3H]C2-3 was about 23, essentially the same as the value of 14 found with the crude preparation.

Another question regarding RNase PH specificity is whether it can act on a tRNA molecule carrying a 3'-terminal phosphate residue. To investigate this point tRNA-CCA-[3H]Cp was generated from the tRNA-CCA-[3H]Cp, substrate by periodate oxidation, and the ability of RNase PH to act on this molecule was determined relative to its action on tRNA-CCA-[3H]C2-3. As shown in Table III, experiment 2, tRNA-CCA-[3H]Cp is essentially inactive as a substrate compared to tRNA-CCA-[3H]C2-3 tested under identical conditions. However, activity could be restored to the 3'-terminated substrate by treatment with alkaline phosphatase which regenerates a 3'-OH group. This finding demonstrates that a 3'-terminal phosphate group prevents RNase PH action on a tRNA substrate.

$K_m$ and $K_{m}$ Measurements for RNase PH—The apparent $K_m$ values for the two substrates of the degradative reaction, P, and tRNA, were determined from double reciprocal plots of degradative activity versus substrate concentration. The apparent $K_m$ value for P, was 2 mM determined at 3 μM tRNA-CCA-[3H]C2-3. The $K_m$ value for tRNA-CCA-[3H]C2-3 was approximately 1 μM, determined at a saturating concentration of P, 20 μM.

To determine the equilibrium between the synthetic and degradative activities, two separate reactions were carried out under the same conditions as described under “Experimental Procedures.” In one, [3H]CDP and unlabeled tRNA were added to monitor the synthetic reaction, and in the other reaction, tRNA-CCA-[3H]C2-3 and P, were used to follow the degradative reaction. Assuming the synthetic reaction is the forward reaction, the $K_m$ value for the two reactions was found to be 0.25. These data indicate that the RNase PH reaction is freely reversible in vitro. Given the $P$, concentrations present in vivo (~1 mM), these findings suggest that the degradative reaction probably is significant in vivo.

Temperature Sensitivity of RNase PH—Samples of RNase PH were diluted to a concentration of approximately 0.015 mg/ml and incubated at various temperatures for 10 min prior to determining the amount of degradative activity remaining. The data in Fig. 2 show that RNase PH was fairly stable up to 45°C, that it lost slightly more than 50% of its activity when heated at 55°C, and that it was totally inactivated when incubated for 10 min at 65°C. Thus, RNase PH is moderately stable to short term heating under these conditions.

Effect of Sulphydryl Reagents on RNase PH—Based on the nucleotide sequence of rph (orfE), RNase PH contains 4 cysteine residues (assuming they are reduced). To determine whether these residues might play a role in RNase PH activity.

![Fig. 1. Effect of enzyme concentration on the degradative and synthetic reactions of RNase PH.](image)

![Fig. 2. Temperature sensitivity of RNase PH.](image)
Can RNase PH Generate Mature 3'-Termini on tRNA?—An important question with regard to the function of RNase PH is whether the enzyme can generate the mature 3' terminus of tRNA when acting on a tRNA precursor. The enzyme was tested for its ability to accept amino acids, increasing to a maximum aminoacylation level of -35% was probably due to tRNA chains that could not be repaired by tRNA nucleotidyltransferase. Shown in Fig. 3 is the effect of increasing amounts of RNase PH on nucleotide removal from the precursor tRNA-CCA-

### TABLE IV

| Sulphydrol reagent | Incubation time | RNase PH | Degradative activity | Activity remaining |
|--------------------|-----------------|----------|----------------------|--------------------|
|                    | min             | pmol     |                      |                    |
| None               | 10              | 68       | 100                  | 100                |
|                   | 60              | 60       | 100                  | 100                |
| NEM                | 10              | 63       | 93                   |                   |
|                   | 60              | 36       | 60                   |                   |
| PMB                | 10              | 14       | 21                   |                   |
|                   | 60              | <5       | <5                   |                   |
FIG. 4. Gel filtration of RNase PH. Purified RNase PH was run on a column of Ultrogel AcA 44 as described under “Experimental Procedures” using four different levels of protein: A, 2 µg; B, 6.5 µg; C, 13 µg; D, 65 µg. Fractions were collected and assayed for both the synthetic and degradative activities using standard conditions, and the time of assay was as noted on the ordinates. For panels A-C, 40- and 25-µl portions were assayed for the synthetic and degradative reactions, respectively, and for panel D, 10 and 5 µl were assayed. The arrows indicate elution positions of the size standards, blue dextran 2000 (2000 kDa); bacterial alkaline phosphatase (80 kDa), and ovalbumin (43 kDa). □, synthetic activity; △, degradative activity.

FIG. 5. Cross-linking of RNase PH. Samples of RNase PH at different protein concentrations were treated with DMS as described under “Experimental Procedures,” and then run on SDS-PAGE. Lane 1, protein standards: trypsin inhibitor, ovalbumin, bovine serum albumin, bottom to top; lane 2, 0.17 mg/ml RNase PH, no DMS; lane 3, 0.17 mg/ml; lane 4, 0.34 mg/ml; lane 5, 0.52 mg/ml; lane 6, 0.69 mg/ml; lane 7, 0.87 mg/ml. Samples in lanes 3–7 were treated with the cross-linking agent. The higher molecular weight bands above RNase PH seen in lane 2 were due to some leakage from lane 1 in this experiment and are not normally present. The lower molecular weight band is seen in the preparation and is due to some degradation of RNase PH (8).

The data presented here show that the OrfE protein has both the degradative and synthetic activities of RNase PH, and they confirm our previous suggestion that orfE (now called rph) encodes this ribonuclease (6). The importance, if any, of RNase PH activity to the functioning of the pyr operon in which it is encoded remains to be established.

The availability of large amounts of highly purified RNase PH has allowed us to carry out a detailed characterization of the enzyme. The properties of the purified enzyme that were retested were essentially the same as determined earlier with
less pure material (5). In addition, much new information that could not be determined with impure preparations was obtained. Most importantly, the highly purified enzyme was shown to be devoid of activity in the absence of P\(_{\text{i}}\), indicating that RNase PH is a strictly phosphorolytic nuclease, and that the low levels of hydrolytic activity observed earlier must have been due to contaminating enzyme(s).

The studies presented here also showed that, as expected for an enzyme with an equilibrium constant close to unity, purified RNase PH can catalyze the synthesis of RNA. However, the unusual activity versus enzyme profile observed for the synthetic reaction remains to be explained. One possibility is that at higher enzyme levels the longer chains synthesized are better substrates for further addition of nucleotides. This effect would not be apparent in the degradative reaction in which the size of the substrate, if anything, decreases. However, it would be consistent with the higher degradative activity on poly(A) compared to tRNA substrates. A second possibility is that the aggregation of RNase PH that should be observed by gel filtration at varying protein concentrations and by cross-linking, RNase PH was shown to aggregate readily to higher order multimers. Whether this aggregation serves any physiological role remains to be determined. Nevertheless, it does explain the discrepancies in molecular weight determinations made by us and Jensen et al. (8). This finding also suggests that the smallest active form observed by gel filtration (45–50 kDa) may be due to a dimer that partially dissociates to monomer during the chromatography. It is also possible that the larger size on gel filtration compared to SDS-PAGE is due to an elongated shape for the monomer.

The preference of RNase PH for tRNA precursor molecules compared to mature tRNA suggests that this enzyme could participate in tRNA processing. Moreover, its ability to generate mature tRNAs that can accept amino acids supports this idea. However, compared to RNase D (13), which carries out a similar reaction, RNase PH is less fastidious with regard to entering the -CCA sequence. Thus, RNase PH generated less mature tRNA compared to RNase D (13), and the amount of mature tRNA produced was increased by the presence of tRNA nucleotidyltransferase. On the other hand, in vivo in the absence of RNase D, RNase PH may play a significant role in the processing of the 3' terminus of tRNA precursors, and this is supported by the inviability of strains lacking RNase PH when this mutation is in combination with other RNase mutations. The ability of RNase PH to act on poly(A) suggests that it may act preferentially on unstructured RNA molecules, and its higher activity on tRNA precursors compared to mature tRNA may reflect the distance of the residues removed from the double-stranded aminocoyl stem of tRNA. The greater difficulty in removing residues as the aminocoyl end is approached, suggests that in vivo final 3' processing of tRNA may not be the primary function of RNase PH, although it may serve as a backup enzyme for this process. The availability of mutants lacking RNase PH, alone or in different combinations with mutants lacking other RNases, should now make it possible to sort out the functions of these multiple E. coli exoribonucleases.

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