The Role of Individual Cysteine Residues in the Activity of Escherichia coli RNase T*

Zhongwei Liţ, Lijun Zhan, and Murray P. Deutscher§

From the Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06030-3305

Escherichia coli RNase T, which is responsible for the 3′ processing and end-turnover of tRNA and the maturation of 5 S RNA, is extremely sensitive to sulfhydryl reagents and to oxidation, suggesting a role for cysteine residues in its activity. Titration of homogeneous RNase T with 5,5′-dithiobis-(2-nitrobenzoic acid) revealed that the 4 cysteine residues present in each of the two protein subunits are in a reduced form and that 1 or 2 of them are important for activity. To identify these residue(s), each of the cysteines in RNase T was changed individually to either serine or alanine. The serine mutant at position 168 is greatly reduced in RNase T activity both in vivo and in vitro; likewise, the serine mutant at position 112 and the alanine mutants at positions 112 and 168 also display decreased RNase T activity. Mutations at the other cysteine positions show little or no change. Kinetic analyses of the mutant enzymes showed that the Kₘ values of C168S and C168A are increased considerably, whereas their Vₘₐₓ values are reduced only slightly compared to the wild type enzyme. The other mutant enzymes are little changed. Additional amino acid replacements at position 168 showed that the in vivo and in vitro activities of RNase T are in the order Cys ~ Val > Ala > Ser ~ Asp > Asp, which closely follows the relative hydrophobicity of these amino acid residues. However, the affinity for tRNA, determined by fluorescence quenching, is not altered in C168S, suggesting that Cys-168 is not directly involved in substrate binding. Interestingly, proteins altered at position 168 showed increased temperature sensitivity as the residue at that position became less hydrophobic. These data indicate that Cys-168 contributes a hydrophobic group that influences the structure and ultimately the catalytic activity of RNase T.

RNase T is one of the eight distinct exoribonucleases known to be present in Escherichia coli (1). The enzyme was originally identified based on its ability to remove the 3′ terminal A and penultimate C residues from tRNA (2). The subsequent isolation of a mutant strain deficient in RNase T (3) demonstrated that this enzyme, together with tRNA nucleotidyltransferase which repairs the -CCA sequence, is responsible for the process of end-turnover of tRNA that is known to occur in vivo in both prokaryotic and eukaryotic cells. Subsequent studies showed that RNase T also participates in the 3′ processing of tRNA precursors, being most effective in removal of the residue immediately downstream of the -CCA sequence (4, 5). Interestingly, although the role of RNase T in maturation of tRNA can be circumvented by the presence of other exoribonucleases (4, 5), cells devoid of RNase T still grow 5–10 min slower than wild type (6), suggesting an additional role for this RNase in RNA metabolism. Very recently, we have shown that RNase T also is required for the maturation of 5 S RNA; no mature 5 S RNA is made in the absence of this enzyme. Rather, a molecule with 2 extra residues at the 3′ terminus accumulates, and this incompletely processed 5 S RNA is incorporated into ribosomes (7). Based on these observations, it is clear that RNase T plays an important role in RNA metabolic processes.

RNase T has been purified to homogeneity (8). The enzyme is an α₂ dimer of approximately 47 kDa. The sequence of the rnt gene encoding RNase T has also been elucidated (9). RNase T is a 3′–5′ exoribonuclease that initiates attack at a free 3′ terminus of tRNA; aminoacyl-tRNA is not a substrate. RNase T is extremely sensitive to sulfhydryl reagents and to air oxidation (8), expecting that one or more of the 4 cysteine residues present in each subunit may be important for the enzyme’s activity. Inasmuch as —SH groups have not previously been implicated in the action of RNases, these observations are of considerable interest.

In this study we use chemical modification, site-directed mutagenesis, and activity measurements in vitro and in vivo to investigate the role of the individual cysteine residues in RNase T. Our data indicate that Cys-168 plays an important role in RNase T activity by contributing a hydrophobic residue that is important for the structure of this enzyme.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Phages—E. coli K12 strain UT481 (Δlac-pro), hsdS(r– m–), lacA, lacZ) was used for routine cloning experiments. Strain Cj 236 (Δlac, ung), thi-1, rElA/pc 105(cmF+) (10) was used to prepare uracil-containing DNA. Strain CA244 (lacZ, trp, rDa, spoT) (11) and its derivatives CA244 (RNase T−), CA244 T− (RNase T−, T−), CA244CCA T− (lacking tRNA nucleotidytransferase and RNase T), CAN20-12EPH− (RNase T−, T−, D−, BN−, PH−) were described earlier (5, 12) and were used for in vitro and in vivo measurement of RNase T activity, and for purification of RNase T. Plasmids pBSI (+) (Stratagene) and pOU61 (13) were used to clone the EcoRI-BamHI fragments containing the wild type or mutant rnt genes (9). A pUC18-rnt construct (9) was used to overexpress RNase T. Phage R408 (14) was used as a helper phage for preparing single-stranded phagemid DNA.

Materials—Affi-Gel blue (100–200 mesh), hydroxylapatite (Bio-Gel HT), and carrier ampholytes were obtained from Bio-Rad. Ultragel AcA44 was from LKB. Sequel for DNA sequencing was purchased from National Diagnostics. The sulfhydryl reagent DTT was obtained from Sigma. Freund adjuvants for raising antibody were purchased from Life Technologies, Inc. Restriction endonucleases, phage

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† Present address: Laboratory of Molecular Psychiatry, Dept. of Psychiatry, Yale University School of Medicine, New Haven, CT 06508.

§ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Miami School of Medicine, P.O. Box 016129, Miami, FL 33101-6129. Tel.: 305-243-3150; Fax: 305-243-3955.

1 Z. Li and M. P. Deutscher, unpublished observations.

2 The abbreviations used are: DTT, 5,5′-dithiobis-(2-nitrobenzoic acid); DTNB, 2,2′-dithiobis-(nitrrobenzoic acid); 2,3-dinitrobenzoic acid. 2,3-Dinitrobenzoic acid; DTT, 5,5′-dithiobis-(2-nitrobenzoic acid).
RESULTS

Purification of Wild Type RNase T—As a first step for studying the importance of cysteine residues of RNase T, a procedure was developed for purifying large amounts of the protein. A cell expressing RNase T due to a multicopy plasmid carrying the rt gene (9) was used as the starting material. The purification procedure followed that previously reported for the endogenous enzyme (8), but one chromatographic step was eliminated. The DTT concentration was increased to 5 mM during the purification and in the assay, and this led to significant stabilization of RNase T. Thus, the final specific activity of the preparation described here is 50% higher than that described previously, even though both preparations were homogeneous.

The homogeneity of the current preparation was demonstrated both by SDS-polyacrylamide gel electrophoresis (Fig. 1, lane 1) and by amino acid composition, which was in close agreement with that predicted from the DNA sequence (data not shown) (9). A summary of the purification procedure is presented in Table I. As observed earlier (8), removal of inhibitors in the S100 fraction leads to a large increase in activity after the first column step. Approximately 25 mg of homogeneous RNase T was obtained from 150 g of wet E. coli cells. The purified protein was used to generate an RNase T-specific antibody as described in “Experimental Procedures.”

Modification of RNase T by DTNB—The role of cysteine residues in RNase T activity was assessed by treatment of the purified enzyme with the sulfhydryl modiﬁer agent DTNB. This reagent reacts with free sulfhydryl groups to form a disulfide with the concomitant stoichiometric release of a colored product that can be measured at 412 nm (ε = 14,150) (21). At each time point of the measurement, the degree of modification by DTNB and the level of RNase T activity were determined. Fig. 2 shows the relationship between the degree of sulfhydryl modification and the activity of RNase T. RNase T activity decreased rapidly in the first few minutes after mixing with concentration.

Situated-directed Mutagenesis—This was carried out by the method of Kunkel (10). Single-stranded uracil-containing DNA from pBS(+(−)rt) was prepared by infection of strain CJ236 containing this phagemid with phage R408. This DNA was used as the template for synthesizing the mutant strand of DNA. After annealing of the appropriate 5′-phosphorylated mutagenic oligonucleotide, the strand was extended and circularized by Sequenase 2.0 and T4 DNA ligase. The resulting plasmid was used to transform strain UT481. Mutant clones were selected randomly and confirmed by DNA sequencing.

Protein Analysis—Protein was measured by a colorimetric method or by A280 absorbance (17, 18). Standard procedures for SDS-polyacrylamide gel electrophoresis and gel staining (19), and immunoblotting and signal detection (20) were followed.

Modification of Antibody against RNase T—For the antibody, 2 μg of total RNase T was used (see below). The antiserum was tested against purified RNase T using dot-blotts and the peroxidase system for detection. Cross-reacting antibodies were removed from the antiserum by treatment with dried CA44. Two antiserum preparations were tested: one a single-copy plasmid vector (10). Titration of the antiserum against RNase T indicated at least a 10-fold increase in RNase T-specific antibody over that of the preimmune serum.

Recombinant DNA Techniques—Isolation of double-stranded and single-stranded DNA, restriction enzyme digestion, ligation, transformation, and DNA sequencing were carried out using established procedures (10).

Electrophoresis was performed using a 12% gel and proteins were visualized after silver staining as described under “Experimental Procedures.” Lane 1, 1 μg of Ultrogel AcA44 peak fraction of wild type RNase T. Lanes 2–5, samples from different steps of C1685 purification in which 0.5 μg of Ultrogel AcA44 column sample (lane 2), 1.1 μg of hydroxylapatite column sample (lane 3), 3.4 μg of Affi-Gel blue column sample (lane 4), or 13.2 μg of S100 (lane 5) were loaded. The positions of the protein size standards are shown on the left.
DTNB, in concert with the increase in the degree of —SH modification. Modification by DTNB reached a maximum in 5–10 min (varies with DTNB concentration), at which point RNase T activity was totally inactivated, indicating that the presence of free —SH groups are essential for RNase T activity. The maximum modification corresponds to close to four —SH groups per subunit of RNase T. Thus, the 4 cysteine residues in each monomer are in a reduced form, and all of them are accessible to DTNB in the native protein. The completeness of the modification was confirmed by the reaction of DTNB with protein denatured with either 7.8 M urea, 6 M guanidinium chloride or 0.5% SDS. In each case the same maximum degree of modification observed with the native protein was seen within 30 s after mixing the denatured proteins with DTNB (data not shown). The slower modification of the native protein suggests that the —SH groups may be partially buried, but still accessible enough to result in complete reaction in a short time. It was not possible to determine from these studies whether some —SH residues might be more reactive. Rather, the —SH residues appear to be close to randomly modified, as the reaction is pseudo first-order and monophasic. Assuming random modification and using the binomial distribution, it is estimated that one or two cysteine residues are important for RNase T activity.

The importance of the cysteine residues was further demonstrated by the complete restoration of RNase T activity upon regeneration of the sulfhydryls with excess DTT (Fig. 2). The half-time of reactivation was about 14 min at 26 °C, much longer than that needed for the half-time of inactivation (1 min), even with a much higher (80-fold) DTT concentration demonstrating the extreme sensitivity of RNase T to oxidation, as noted earlier (8). The reactivation by DTT indicates that the loss of RNase T activity was caused solely by the formation of disulfide bonds, and not by any irreversible changes in the protein structure. However, by these methods it was not possible to distinguish whether reduction of a particular modified —SH had more of an effect than others. Thus, other studies, such as site-directed mutagenesis, were necessary to determine which cysteine residues are most important for RNase T activity.

Site-directed Mutagenesis of RNase T—Conversion of each of the 4 cysteine residues to other amino acids was performed with the phagemid pBS(+)-rnt as described in “Experimental Procedures.” The changes made are shown schematically in Fig. 3A. The nucleotide replacement(s) for each mutant were confirmed by DNA sequencing. In addition, to ensure that no changes occurred in nucleotides in other parts of the rnt gene, the mutant clones were checked by DNA sequencing of the whole gene and/or by recloning of the mutated fragments into the wild type gene. Cloning of the mutant rnt genes into the single copy plasmid pOU61 was confirmed by DNA restriction fragment analysis.

In most cases, the replacement of a cysteine residue by another amino acid had little effect on the amount of RNase T protein present in the mutant clone as compared to the wild type clone. This was determined by immunoblotting of extracts from cells containing the single-copy plasmid (not shown) or the multicyclic plasmid (Fig. 3B). However, considerably less RNase T protein was repeatedly observed with the C168D mutant (~10%) (lane 14), and a small decrease was observed with the C112S mutant with the single copy plasmid (data not shown). No RNase T protein is present in the host cell used (lane 2).

Cys-112 and Cys-168 Substitutions Affect RNase T Activity—We initially examined the effect on RNase T activity of a serine or alanine substitution at each cysteine residue. A serine substitution would be expected to retain some of the nucleophilicity of the —SH group, whereas this would be completely lost with an alanine substitution. The results are summarized in Table II. In vitro measurements of RNase T activity were carried out in most cases with sonicated cell extracts from strain CA244T containing either the wild type or mutant rnt gene on the single copy plasmid pOU61. However, for more accurate determination of the relative activities of C168S, C168N, and C168D, extracts from cells containing multicopy plasmids were used. This assay is specific for RNase T. With vector alone, less than 1% of wild type activity is observed. As compared to the wild type gene, the serine mutant at position 168 (C168S) displayed a ~95% decrease in RNase T activity; however, the alanine substitution was lowered only ~65%. This observation suggests that although Cys-168 is important for RNase T activity, it is most likely not the nucleophilicity of the —SH group that is the major factor. Substitutions of alanine and serine at position 112 also affect RNase T activity; however, the latter case is more consistent with the primary effect on RNase T activity (Table II).

RNase T activity also can be estimated in vivo. We have
individually into strain CA244CCA1130.

1. **Cys Residues and RNase T Activity**

   The close agreement between the in vitro and in vivo RNase T activities strongly supports the conclusion that the cysteines at positions 112 and 168 are important for RNase T activity, whereas those at the other two positions are not. Thus, modification of residues 112 and 168 by DTNB may account for the loss of RNase T activity observed upon treatment with this reagent. Interestingly, however, the substitutions at positions 112 and 168 behave very differently. At position 112, the serine substitution displays more than twice the activity of the alanine substitution (considering the lowered RNase T protein for C112S), whereas at position 168 alanine is approximately 10-fold more active than serine.

   It should also be noted that the clone with the C168S substitution actually grows even more rapidly than the cell with the vector alone (Table I), with visible colonies appearing after 18 rather than 24 h. We attribute this difference to the fact that RNase T− cells grow more slowly than wild type (6), and that a small amount of RNase T may help to overcome that growth defect, yet still be low enough not to have a significant negative effect on the growth of CCA− cells.

   Kinetic Parameters of the Cysteine Substitution Mutants—To further explore the effects that the cysteine substitutions have on RNase T catalytic properties, the Km and Vmax values were determined for each of the mutants (Table II). The Vmax values were corrected for the amount of RNase T present as determined by immunoblotting. As shown in Table II, the wild type enzyme has a Km value of 3.9 μM and a Vmax of 3.6 unit/mg. Alteration of the cysteine residue at position 168 led to increasing Km values as the residue was changed to valine, alanine and serine. On the other hand, the changes in Vmax were less dramatic. Alterations at position 112 led to relatively small changes in Km or Vmax, and there was essentially no change in these parameters with the changes at the other cysteine positions.

   To ensure that the Km increase observed in the C168S done was really due to the mutant enzyme, and not to any problems due to using extracts, the mutant RNase T was also purified to homogeneity (Fig. 1 and Table III). The specific activity and overall purification were apparently much lower for the mutant than for the wild type enzyme, but this was due to considerable inactivation of the mutant protein during the purification procedure. As a consequence, Vmax values for this enzyme are not meaningful. However, the apparent Km values for the homologous wild type and mutant enzymes were 3.0 and 41 μM, respectively, in close agreement with those determined in extracts (Table II).

   To examine whether the C168S mutant is affected in tRNA substrate binding, we have directly measured the binding of substrate to each of the purified enzymes by determining the fluorescence quenching of increasing concentrations of E. coli tRNA-C-C-A. These data, determined at two temperatures, are presented in Table IV. There is very little difference in tRNA affinity between the C168S mutant and wild type RNase T suggesting that Cys-168 is not required for binding of substrate. Thus, the lowered activity of the C168S mutant is not a consequence of a change in binding affinity. Likewise, the increased Km of the C168S protein is not due to altered substrate binding.

   Hydrophobicity at Position 168 Influences RNase T Activity—The finding that the C168A derivative of RNase T is close to 10-fold more active than the C168S mutant demonstrated that it is not the nucleophilic properties of the cysteinyl—SH group that are of importance. Rather, it appeared that the hydrophobic properties of the—CH2SH moiety might be the determining factor. This conclusion was supported by the observation that a
Cysteine substitution mutants of RNase T: expression and activity in vitro and in vivo

| Protein expressed | Wild type | C11A | C11S | C112A | C112S | C168V | C168A | C168S | C168N | C168D | C195A | C195S | Vector |
|-------------------|-----------|------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| CCA T cell growth | +         | +    | +    | +     | +     | +     | +     | +     | +     | +     | +     | +     | +      |
| Multiplicy T      | –         | –    | –    | –     | –     | –     | –     | –     | –     | –     | –     | –     | –      |
| Single copy T     | –         | –    | –    | –     | –     | –     | –     | –     | –     | –     | –     | –     | –      |
| Relative activity (%)<sup>b</sup> | 100 | 116 | 73 | 21 | 40 | 107 | 36 | 4 | <1 | <1 | 86 | 73 | (1) |
| K<sub>v</sub> (um)<sup>a</sup> | 3.9 | 4.0 | 3.8 | 6.6 | 3.3 | 7.0 | 13 | 51 | ND<sup>a</sup> | ND<sup>a</sup> | 5.1 | 5.1 | ND    |
| V<sub>max</sub> (unit/mg)<sup>a</sup> | 3.6 | 3.9 | 3.3 | 1.3 | 4.8 | 7.6 | 3.1 | 1.3 | ND<sup>a</sup> | ND<sup>a</sup> | 2.8 | 3.4 | ND    |

<sup>a</sup> ND, not determined.
<sup>b</sup> Activity was assayed under standard conditions using sonicated extracts of CA244<sup>++</sup>T cells containing rnt on a single copy plasmid except for C168S, C168N, and C168D in which the multicopy plasmid was used. Results are expressed as a percentage of wild type activity with each plasmid. Values shown are the average of at least two independent experiments, each carried out in duplicate. The very low activity found with the vector alone (1%) has been subtracted from each value.

Valine mutant is even more active than the alanine mutant, and as active as the wild type enzyme (Table II). Moreover, the more polar amino acids, asparagine and aspartic acid, show very little RNase T activity. In fact, there is a very strong correlation between RNase T activity and the hydrophobicity of the different amino acids at position 168. The activity of the various RNase T molecules is in the order Val (> Asp (0.987) > Ala (0.702) > Ser (~ 0.453) > Asn (~ 1.003) ~ Asp (~ 1.935) (based on a combination of the in vitro and in vivo assays) and follows the hydrophobicity values (22) shown in parentheses very closely. These data strongly suggest that the role of cysteine at position 168 is to contribute a hydrophobic side chain.

Mutations at Position 168 Render RNase T Temperature-sensitive—We noted earlier that RNase T with a C168S change loses considerable activity during purification. This suggested that alterations at position 168 may lead to inactivation of RNase T under mild conditions. To test this, cell extracts from the wild type, and the C168V, C168A, and C168S clones were preincubated at 37 °C, and at different time points samples were assayed for RNase T activity. As shown in Fig. 4A, wild type RNase T loses 25% of its activity after 5 min of incubation, indicating some degree of thermostability. However, the mutants are inactivated considerably more rapidly. The serine mutant is extremely sensitive to incubation at 37 °C, with a half time of inactivation of <1 min. The thermostability of the various derivatives at position 168 are in the order of Cys > Val > Ala > Ser, in close agreement with the order for activity. The inactivated proteins are not degraded during preincubation, as measured by immunoblotting (data not shown).

Temperature sensitivity of the mutant was also observed when the purified wild type and C168S RNase T proteins were incubated at 37 °C. C168S RNase T is much more thermostable than the wild type enzyme (Fig. 4B). However, the thermostability of the mutant protein can be significantly alleviated by the presence of tRNA (Fig. 4B). This is consistent with the observation that the C168S derivative is somewhat more stable in a cell extract than in purified form.

The temperature sensitivity of the various RNase T derivatives obviously contributes to their lowered activity when assayed at 37 °C. The influence of temperature was clearly shown when the activity of the C168S enzyme was compared to that of the wild type RNase T at different temperatures (Fig. 5). The relative activity of the mutant RNase T decreases progressively from ~ 65% at 16 °C to ~ 5% at 44 °C. These data show that replacement of the cysteine residue at position 168 dramati-
that this residue is important for the cysteine side chain. We show in a companion study (28) that this residue participates directly in catalysis because even with an alanine substitution, at least 20% of RNase T activity remains. It is an interesting question as to why position 168 evolved to be a cysteine residue when its hydrophobic properties seem most important. In fact, valine in this position leads to a slightly more active enzyme. On the other hand, the valine derivative is more thermosensitive. Clearly, other factors such as side chain size or hydrogen bonding must also contribute to the structural properties required at this position. It is also possible that transient intersubunit disulfide formation involving Cys-168 could contribute to stability. Structural analysis by x-ray crystallography or NMR spectroscopy will be needed to resolve this question. It is interesting that a cysteine residue in a DNA binding domain, that of the Myb protein, also appears to function by virtue of its hydrophobicity (29).

A puzzling observation with regard to the substitutions at position 168 is that as the residue at this position becomes less hydrophobic, the $K_m$ value for tRNA increases. The fluorescence quenching experiments clearly showed that this is not an effect on substrate binding, so that some other factor must be involved. Inasmuch as these amino acid substitutions render RNase T temperature sensitive, and the tRNA substrate serves to protect the enzyme against inactivation, we suspect that we may simply be observing increased stability of the mutant RNase T as the tRNA substrate concentration is increased. What appears to be increasing activity with increasing substrate concentration may only represent more active enzyme present during the assay. In fact, increasing the tRNA concentration was found to lead to increasing thermostability of the mutant enzyme during the assay, and consequently, the activity of RNase T.

**DISCUSSION**

Although cysteine residues are known to participate in protein-RNA interactions through Michael adduct formation (23–25) or through metal binding (26, 27), these residues have not previously been implicated in RNase activity. Here we show that 2 of the 4 cysteine residues of RNase T play an important role in the activity of this enzyme. Cys-168, in particular, has a dramatic influence on RNase T stability and hence, on RNase T activity, and functions by virtue of the hydrophobic properties of the cysteine side chain. We show in a companion study (28) that this residue is important for $\alpha_2$ dimer formation by RNase T. The second cysteine residue, Cys-112, has less of an effect on RNase T activity, but in this case, the nucleophilic properties of the —SH group appear to be paramount. However, it is unlikely that this residue participates directly in catalysis because even with an alanine substitution, at least 20% of RNase T activity remains.

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