It was shown that Cys-168 is required for RNase T function and thermostability and that its hydrophobic properties are important for this role (Li, Z., Zhan, L., and Deutscher, M. P. (1996) J. Biol. Chem. 271, 1127-1132). To understand the molecular basis for these findings, further studies of Cys-168 and RNase T structure were carried out. Treatment of RNase T with the sulfur-dryl-modifying agent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) leads not only to inactivation, but also to monomerization of the protein. Similarly, specifically converting Cys-168 to either serine or asparagine leads to loss of activity and to monomer formation at 37 °C. However, at 10 °C the serine mutant remains as a dimer and retains full RNase T activity, whereas the asparagine derivative shows only a low level of activity and of dimer formation. These data show a strong correlation between activity and the dimeric form of RNase T. The importance of dimer formation was also shown in vivo using genetic studies. An inactive mutant of RNase T, termed HA2, which exists as a dimer at 37 °C in vitro, completely suppresses endogenous RNase T activity in vivo and in vitro when introduced into a RNase T+ cell on a multi-copy phagemid, most likely as a consequence of inactive heterodimer formation. Introduction of the HA2 gene on a single-copy plasmid, as expected, leads to a proportionally smaller effect on endogenous activity. The dominant negative effect displayed by the HA2 protein can be relieved by an additional mutation in HA2 RNase T that abolishes its ability to dimerize. An inactive mutant asparagine derivative of Cys-168, which also does not dimerize, also shows little of the dominant negative phenotype. Thus, these data demonstrate that RNase T dimerizes in vivo, that the dimer form is required for RNase T activity, and that Cys-168 is needed for dimerization of the enzyme.

RNase T, one of eight exoribonucleases identified in Escherichia coli, plays an important role in both tRNA and 5 S RNA metabolism (1-3). In a companion study (4), we showed, by chemical modification, site-directed mutagenesis, and activity measurements in vivo and in vitro, that the enzyme contains an essential cysteine residue at position 168, as well as a second cysteine at position 112 that also contributes significantly to RNase T function. Interestingly, Cys-168 does not appear to participate directly in substrate binding or catalysis. Rather, this residue contributes a hydrophobic group that is important for RNase T structure, affecting the protein's thermostability and ultimately its activity.

In this study, we examine the structural role of Cys-168 in more detail. Our data demonstrate that treatments which modify or alter this residue convert RNase T to an inactive monomer, and that restoration of activity is related to dimer formation. Moreover, inactive RNase T can display a dominant negative effect in vivo, but only if the mutant protein is capable of dimerizing. These findings indicate that Cys-168 is involved in the dimerization of the RNase T subunits and that dimer formation is required for RNase T activity.

EXPERIMENTAL PROCEDURES

Materials—The bacterial strains, plasmid, and phages used are as described here and in a companion study (4). Likewise, the reagents used in the experiments reported here have been described (4).

Methods—Assays for RNase T activity, protein quantitation, immunoblotting, recombinant DNA procedures, and DNA sequencing were all carried out as previously described (4).

In Vitro Random Mutagenesis of the rnt Gene—Mutagenesis of the rnt gene present in a phagemid was carried out by a modification of the procedure previously described for naked DNA (5). The single-stranded phagemid pBS(+) carrying the wild type rnt gene was produced by infecting strain CA244 harboring the phagemid with the helper phage R408. Phagmid particles (1 × 10⁹ colony-forming units in 0.7 ml of 2 × YT medium) were mixed with 0.4 ml of 0.5 M KPO₄ (pH 6.0), 5 mM EDTA, and 0.8 ml of 7% NH₄OH-HCl in 0.45 M NaOH. The mixture was incubated for up to 150 min at room temperature, and the treatment was stopped by dilution at 1:100 into yeast extract, trypotone medium. The mutagenized phagemid preparation was introduced into strain CA244 CCA by mixing with an equal volume of culture containing cells in excess and incubating for 20 min at 37 °C. Infected cells were spread on YT medium containing ampicillin (50 μg/ml), and resistant colonies were screened for RNase T activity by assay. The rnt gene from identified RNase T+ mutants were then recloned into untreated vectors to eliminate any possible effects of mutated vectors.

Gel Filtration of Cell Extracts and Detection of RNase T—Cells were grown in YT medium to an A₅₉₀ = 1, and S100 extracts were prepared as previously described (4). Columns of Ultrogel AcA44 were equilibrated with buffer A (10 mM Tris-Cl, pH 7.5, 5 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM KCl, 10% glycerol) at the indicated temperature. The S100 extracts were adjusted to 1 M KCl prior to addition to the column. Fractions were collected, and the elution position of RNase T was determined by a dot-blot assay using RNase T antibody coupled with a peroxidase-labeled secondary antibody (4).

RESULTS

DTNB Modification of RNase T Leads to Monomer Formation—Treatment of RNase T with DTNB results in covalent modification of all the cysteine residues in the protein and complete loss of RNase T activity (4). To ascertain whether this modification affects the structure of RNase T, S100 extracts from a strain overexpressing the enzyme were subjected to gel

The abbreviations used are: DTNB, 5,5’-dithiobis-(2-nitrobenzoic acid); PAGE, polyacrylamide gel electrophoresis.
filtration on Ultrogel AcA44, and the location of RNase T was determined by immunoblotting (Fig. 1). Untreated RNase T protein elutes as a symmetrical peak at a position corresponding to a globular protein with a size of approximately 50 kDa, in agreement with that previously determined for the dimeric form of the enzyme (6). In contrast, DTNB-treated RNase T elutes as two peaks; the major one elutes later than dimeric RNase T, indicating that it is of smaller size. SDS-PAGE of the RNase T in this peak, followed by immunoblotting, showed that it is not degraded (Fig. 2). This, coupled with its elution position upon gel filtration, demonstrates that DTNB treatment converts a major portion of RNase T to the free monomer. Thus, the Cys residues of RNase T are involved in some manner in dimerization of the protein.

The second RNase T peak generated by DTNB modification actually is slightly larger than the dimeric form of the enzyme (Fig. 1). SDS-PAGE and immunoblotting of the material in this peak indicated that the RNase T polypeptide present also is of normal size (Fig. 2). However, SDS-PAGE run under nonreducing conditions revealed that RNase T is also present in several bands of various sizes that are larger than the dimeric form of the enzyme (data not shown). We suspect that these represent cross-linked products between RNase T and other proteins that form by disulfide interchange with the DTNB-modified protein.

Mutations at Cys-168 Result in Monomers—In our companion study (4), we showed that the RNase T proteins resulting from mutations at position 168 displayed increased temperature sensitivity. To determine whether this effect also might be due to the dimerization state of RNase T, we have examined several of the RNase T mutant proteins by gel filtration at different temperatures. The three mutants studied bear either a serine, an asparagine, or a valine substitution at position 168. The C168S mutant retains ~5% of RNase T activity under standard assay conditions, and it rapidly loses this residual activity upon incubation at 37 °C. The C168N mutant is essentially inactive under standard assay conditions (4). The valine mutant is fully active, but it is somewhat more temperature-sensitive than the wild type enzyme (4).

As noted above (Fig. 1), wild type RNase T elutes at the dimer position at 37 °C, and as shown in Fig. 3A, it elutes at the identical position at 10 °C. In contrast, the C168S mutant protein elutes at the monomer position at 37 °C, but it elutes at the dimer position at 10 °C (Fig. 3B). At 20 °C the protein elutes in between the monomer and dimer position, undoubt-
the protein is a monomer.

Study of the C168N mutant protein leads to the same conclusion, i.e. dimerization is required for activity. Gel filtration at 37 °C, at which the enzyme is inactive, shows only the monomer form (Fig. 3C). Even at 10 °C, the C168N mutant elutes primarily between monomer and dimer, with at most a small shoulder extending into the dimer position. For this mutant protein, RNase T activity at 10 °C is at most ~25% of wild type. Thus, the C168N mutant, which contains a more hydrophilic substitution at position 168, and is less active at each temperature, also is more temperature-sensitive for dimerization. The valine mutant protein, on the other hand, runs on gel filtration as the dimer form at 10, 20, and 37 °C (data not shown). These data support the conclusions that the Cys residue at position 168 participates in dimer formation, that the hydrophobicity of the residue at this position is important, and that RNase T activity depends on the dimer form of the enzyme.

Evidence for RNase T Dimerization in Vivo—To determine whether RNase T dimerizes in vivo and whether both subunits need to be active for RNase T to function, we have examined whether a plasmid-borne rnt mutant gene would exert a dominant negative effect on wild type RNase T expressed from the chromosome. To do this, an inactive RNase T mutant was constructed by random mutagenesis of a rnt gene cloned in the phagemid pBS (+). The details of the method are described under "Experimental Procedures," but it should be noted that the single-stranded phagemid DNA present in the phage particle provides a useful starting material for in vitro mutagenesis, and can be conveniently and efficiently introduced into F + cells by infection. This represents a major improvement over current methods which use isolated DNA and cell transformation.

Screening of RNase T+ clones was carried out in CCA- cells that lack the enzyme tRNA nucleotidyltransferase, as described in our companion study (4). In a CCA- background, lowered RNase T activity leads to faster growth. Thus, after phagemid infection, the largest colonies were assayed for RNase T activity in vitro. By this method, one mutant, termed HA2, with very low RNase T activity, was selected. Based on DNA sequencing (data not shown), the HA2 rnt gene contains two mutations that would lead to Arg-15 → His and Gly-28 → Arg in RNase T. The HA2 protein is normal with regard to its amount and size (see Fig. 2; as noted in the legend, twice as much HA2 protein was loaded), and it exists as a dimer at 37 °C (Fig. 4). However, the HA2 protein is essentially devoid of RNase T activity. In a RNase T- cell, the HA2 gene in the multicopy vector pBS (+), results in a level of RNase T activity equivalent to only 13% of that present in a RNase T+ cell containing the single chromosomal copy of rnt (Table I); no RNase T activity is detectable when the HA2 gene is introduced into a RNase T- cell on the single copy plasmid pOU61 (Table I). Thus, the HA2 gene results in a RNase T with considerably < 1% of wild type activity.

Of most interest, however, is that the presence of this HA2 protein exerts a dominant negative effect on the endogenous RNase T activity present in the RNase T+ cell (Table I). Thus, when HA2 is present on pBS (+), it completely suppresses the activity arising from the chromosomal rnt gene (Table I), and all that is seen is the 13% activity due to the HA2 protein. Likewise, when HA2 is present on pOU61, it decreases RNase T activity over 70%. These findings strongly suggest that the HA2 protein must form a heterodimer with the wild type RNase T monomer resulting in an inactive protein. Indeed, the level of RNase T activity remaining (28%) when HA2 is introduced into the RNase T- cell via pOU61, is just what would be expected for the amount of wild type dimer that should be formed (33%) upon random association of monomers, considering that pOU61 is present at ~2 copies/cell (4).

The dominant negative effect of the HA2 protein can also be examined in vivo by determining how it influences the growth rate of a CCA- T+ cell. It has already been noted that the growth of CCA- cells is exquisitely sensitive to the level of RNase T activity present (4). As can be seen in Table II, introduction of the HA2 gene into a CCA- T+ cell has little effect on growth, whether present on pBS (+) or pOU61. However, introduction of HA2 into the CCA- T- cell leads to much faster growth, and the effect is greater when HA2 is present on the multicopy plasmid. These data show that in vivo the presence of the HA2 protein greatly suppresses RNase T activity, in agreement with the in vitro measurements.

Relation of Dominant Negative Phenotype and Ability to Form Dimers—To further demonstrate that the dominant negative phenotype exhibited by the HA2 protein is due to its association with wild type RNase T monomers and the formation of inactive heterodimers, the HA2 rnt gene was subjected to further mutagenesis followed by screening in CCA- T+ cells for slow growing colonies and assaying for increased RNase T activity. One mutant, HA2-1, was selected based on these criteria. The HA2-1 protein contains a Gly-206 → Ser change in addition to the two changes already present in HA2. Immunoblotting demonstrated that the protein is of normal size; however, it is present at only ~25% of the wild type amount. HA2-1 displays no RNase T activity on its own (Table I) and runs primarily as the monomer upon gel filtration at 37 °C (Fig. 4).

As shown in Table I, the HA2-1 protein shows much less of
RNase T Functions as a Dimer Dependent on Cys-168

**TABLE II**
Effect of plasmid-borne RNase T mutant genes on growth rate of CCA T+ cells

| Plasmid present       | Doubling timea in CCA T+ host | Doubling timea in CCA T+ host |
|-----------------------|-------------------------------|-------------------------------|
| pBS(+)                | 88                            | 57                            |
| pBS(+)–HA2            | 35                            | 50                            |
| pBS(+)–HA2–1          | 65                            | 58                            |
| pBS(+)–C168N          | In viable                     | 88                            |
| pOU61b                | 85                            | 72                            |
| pOU61-HA2b            | 48                            | 70                            |

a Data are based on two or three independent measurements.
b Cells with pOU61 plasmids were grown at 32°C.

Discussion

The data presented in this study relate the dimerization state of RNase T protein, RNase T activity, RNase T thermostability, and the Cys residue at position 168. RNase T normally is isolated as a dimer, and the information presented here indicates that it also exists as a dimer in vivo. Moreover, there is a strong correlation between the dimer form of RNase T and its catalytic activity. Treatments or alterations which were shown to lead to a loss of RNase T activity (4), also result in dissociation of RNase T to the monomer form. Thus, DTNB modification or conversion of Cys-168 to hydrophilic residues, each of which causes loss of RNase T activity, also leads to monomerization of the protein. Most convincingly, lowering the temperature to which RNase T is exposed, stabilizes the dimer form to different degrees depending on the mutant, and results in a concomitant retention of catalytic activity. These findings strongly support the conclusion that it is the dimeric RNase T that is the catalytically competent form.

This conclusion is strengthened by the data indicating that the dimer is present in vivo. Many RNase T mutants when present on the multicopy phagemid pBS(+) lead to faster growth when introduced into CCA T+ cells, implying a reduction in the endogenous RNase T activity by the excess of inactive enzyme (data not shown). With one mutant, HA2, endogenous RNase T activity can be completely suppressed, and the degree of suppression depends on the amount of mutant RNase T made. These data are most consistent with the formation of heterodimers between wild type monomers and the excess of mutant monomers present. Moreover, when the ability of the mutant to dimerize is lost, such as with the HA2-1 mutant, its dominant negative effect also is greatly decreased. These data, which agree completely with the in vitro gel filtration results, indicate that dimers form in vivo as well.

What is the role of Cys-168 in the dimerization process? Clearly, this residue is important because its modification by DTNB or its alteration by mutagenesis can result in monomerization. On the other hand, other hydrophobic residues, such as Val or Ala, also retain more or less activity, and presumably can maintain the dimeric state. Yet, based on the temperature sensitivity of their RNase T activity (4), these alternate residues at position 168 lead to somewhat less stable dimers. Perhaps, a Cys residue at this position can lend to transient disulfide formation with the corresponding Cys on the other monomer which can help to stabilize the dimer. Analysis of the RNase T sequence by the methods of Chou and Fasman (7) and Garnier et al. (8) suggest that Cys-168 is located near the end of a region (residues 151–168) of RNase T rich in hydrophobic moieties. Helical wheel analysis shows that Cys-168 is part of a hydrophobic helical face that conceivably could function as a dimerization domain (Fig. 5). Additional mutations in this region might be useful for addressing this question. Interestingly, another mutation, Gly 206 — Ser, which is present in HA2-1, also affects dimer formation in the background of the other HA2 changes. Whether residue 206 is close in the three-dimensional structure to the hydrophobic region encompassing Cys-168 remains to be determined.

The studies presented here raise some interesting points regarding the active site of RNase T. The dominant negative phenotype exhibited by certain of the mutants suggest that both subunits must be active in order to generate a functional RNase T and that RNase T monomers are not active. One possibility to explain these observations is that both subunits contribute to a single binding site for tRNA. In support of this suggestion is the finding presented in our companion study (4) that tRNA stabilizes the C168S mutant against temperature...
inactivation. Since the serine mutant monomerizes at 37°C (Fig. 3), and the dimer is needed for RNase T activity, this observation suggests that tRNA maintains the enzyme in the dimer form. This would also explain the binding of tRNA to the C168S mutant at 37°C determined by fluorescence quenching, shown in our companion study (4). Thus, in a situation in which the monomer and dimer forms of RNase T are in equilibrium, binding of tRNA only to the dimer form would drive the protein in that direction. This conclusion also predicts that the monomer would bind tRNA weakly, or not at all. In fact, the C168N mutant, which is largely monomeric even at low temperatures (Fig. 3), binds relatively poorly to Affi-Gel blue. Affi-Gel blue is known to strongly bind tRNA binding proteins (9). The fact that the C168N mutant, which is a monomer, binds to this matrix considerably more weakly than either the wild type or C168S mutant protein, is consistent with poor nucleic acid binding by the monomer.

On the other hand, because the HA2 mutant is inactive, but still dimerizes, a functional active site is not simply generated by dimerization. It is possible, but less likely, that each subunit contains its own tRNA binding site, and that correct interaction between the two subunits is necessary to render these sites active. Clearly, further structural work on this important RNA processing enzyme will be necessary to resolve these points.

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