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Role of cysteine residues in ribonuclease H from *Escherichia coli*

Site-directed mutagenesis and chemical modification

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The role of the three cysteine residues at positions 13, 63 and 133 in *Escherichia coli* RNAase H, an enzyme that is sensitive to *N*-ethylmaleimide [Berkower, Leis & Hurwitz (1973) J. Biol. Chem. **248**, 5914–5921], was examined by using both site-directed mutagenesis and chemical modification. Novel aspects that were found are as follows. First, none of the cysteine residues is required for activity. Secondly, chemical modification of either Cys-13 or Cys-133 with thiol-blocking reagents inactivates the enzyme, but that of Cys-63 does not. Thus the sensitivity of *E. coli* RNAase H to *N*-ethylmaleimide arises not from blocking of the thiol group but from steric hindrance by the modifying group incorporated at either Cys-13 or Cys-133.

INTRODUCTION

RNAase H is an enzyme that degrades specifically RNA moieties of a DNA–RNA hybrid (Crouch & Dirksen, 1982). The enzyme has been found ubiquitously in various organisms, including *Escherichia coli* (Miller *et al.*, 1973; Berkower *et al.*, 1973). The physiological function of the enzyme remains obscure, although the importance of the enzyme from *E. coli* for the initiation of the ColE1 replication at the precise position (*ori*) has been demonstrated both *in vitro* (Itoh & Tomizawa, 1980) and *in vivo* (Dasgupta *et al.*, 1987). Because of its wide distribution and unique substrate-specificity, it is believed that the enzyme might participate in important events in cells such as elimination of the primer RNA for DNA synthesis during DNA replication. The structural gene (*rnh*) for *E. coli* RNAase H has been cloned (Horiuchi *et al.*, 1981) and sequenced (Kanaya & Crouch, 1983; Maki *et al.*, 1983). According to the amino acid sequence predicted from the DNA sequence, it is composed of a single polypeptide chain with 155 amino acid residues.

The enzyme contains three cysteine residues and behaves as a monomer (Kanaya *et al.*, 1989). It has been previously reported that this enzyme requires reducing reagents such as dithiothreitol or 2-mercaptoethanol for maximal activity and is inhibited by *N*-ethylmaleimide (NEM) (Berkower *et al.*, 1973). This evokes the question as to whether any of these cysteine residues is essential for the catalytic activity or if alkylation of these cysteine residues with NEM introduces a steric deformation that leads to inactivation of the enzyme. It seems unlikely that a cysteine residue is essential for the catalytic activity, because none of the cysteine residues is conserved in various RNAase H-related sequences, including retroviral reverse transcriptases (Johnson *et al.*, 1986; Doolittle *et al.*, 1989). If a cysteine residue were involved in an active site of the enzyme, it would be expected that this cysteine residue would be conserved in these sequences. Thus it is more likely that the NEM-sensitivity of this enzyme is due to a steric effect of the modifying group incorporated into the cysteine residues. However, hitherto there has been no experimental evidence showing that the cysteine residues in this enzyme are modified with NEM. The NEM-sensitivity of the enzyme has simply been examined by comparing the enzymic activity determined in the presence of NEM with that determined in the absence of NEM. Therefore the possibility cannot be ruled out that modification of amino acid residues other than cysteine residues with NEM causes the inactivation of the enzyme, or that NEM simply acts as an inhibitor for the enzymic activity instead of as a thiol-blocking reagent. Physicochemical characterization of the modified enzyme is required to eliminate such ambiguities.

In addition to the *E. coli* enzyme, many cellular RNAases H from lower and higher eukaryotes have been reported to be inhibited by NEM (Crouch & Dirksen, 1982; Kane, 1988; Vonwirth *et al.*, 1989). Therefore NEM-sensitivity has been often employed to categorize an enzyme that hydrolyses the RNA portion of DNA–RNA hybrid as an RNAase H. However, there are no reports that describe the biochemical characterization of RNAases H treated with thiol-blocking reagents. This is mainly due to the low content of the enzyme in cells and the difficulty in purifying the protein in an amount sufficient for biochemical studies.

E. coli RNAase H has been overproduced in *E. coli* N4830-1 harbouring plasmid pPL801, in which the *rnh* gene is under the control of the P_L promoter (Kanaya *et al.*, 1989), and in *E. coli* JM109 (AK101) harbouring plasmid pDR600, in which the *rnh* gene is under the control of the tac promoter (Kanaya *et al.*, 1990). The availability of sufficient enzyme permits us to examine the structural or functional role of the cysteine residues of this enzyme by chemical modification with various thiol-blocking reagents. Furthermore, the availability of the cloned *rnh* gene permits site-directed mutagenesis to change each of the cysteine residues to determine their importance in the enzymic activity and/or function.

Our major interest is to determine the mechanism by which *E. coli* RNAase H recognizes the DNA–RNA hybrid as a substrate. The three-dimensional structure of the enzyme is obviously required for the complete understanding of the recognition of the substrate. We have already succeeded in obtaining crystals (Kanaya *et al.*, 1989). In parallel with the X-ray analysis, we have utilized site-directed mutagenesis to clarify the structural and functional role of various amino acid residues. Studies on the role of the cysteine residues of *E. coli* RNAase H are part of an effort to identify the active-site residue of this enzyme.

In the present study the role of the cysteine residues in *E. coli* RNAase H has been analysed by employing both chemical modification and site-directed mutagenesis. We report here that the NEM-sensitivity of *E. coli* RNAase H arises not from

Abbreviations used: NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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blocking of the thiol group but from steric hindrance by the modifying group incorporated at either Cys-13 or Cys-133.

EXPERIMENTAL

Cells and plasmids

E. coli strains JM101 and JM109 were obtained from Toyobo Co. (Osaka, Japan), and strain TG-1 was from Amersham International (Amersham, Bucks., U.K.). The *rnh*-deficient strain AK101 of JM101 origin in which the *cat* gene was inserted into the *rnh* gene was constructed as described previously (Kanaya & Crouch, 1984). Plasmid pDR600 in which the *rnh* gene was under the control of the tac promoter and replication-form DNA M13mp19 (rnh) in which the *rnh* gene was inserted in the multicloning site of M13mp19 were constructed in our laboratory (Kanaya *et al.*, 1990). Cells were grown in Luria broth medium (Miller, 1972), with or without antibiotics (100 mg of ampicillin/l and 30 mg of chloramphenicol/l).

Materials

Restriction and modifying enzymes were from either Toyobo Co. or Takara Shuzo Co. (Kyoto, Japan), except *Sst*I and *Sst*II, which were from Bethesda Research Laboratories (Bethesda, MD, U.S.A.). Radioactive compounds were purchased from Amersham International. Isopropyl β-D-thiogalactopyranoside and NEM were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) was obtained from Nakalai Tesque (Kyoto, Japan). Lysyl endopeptidase and iodoacetic acid were from Wako Pure Chemical Industries (Osaka, Japan). PD-10 was from Pharmacia (Uppsala, Sweden). Other chemicals were of reagent grade.

Construction of plasmids

All DNA fragments, generated by hydrolysis with restriction enzymes, were purified by agarose-gel electrophoresis followed by DEAE-cellulose (DE-52) column chromatography before ligation. The ligation reaction was carried out with the DNA Ligation Kit from Takara Shuzo Co. according to the method recommended by the supplier. *E. coli* JM109 was transformed with plasmid as described by Maniatis *et al.* (1982). Selection was for ampicillin-resistance. Plasmid DNA from Amp^r transformants was isolated by small-scale plasmid preparation (Ish-Horowicz & Burke, 1981) and analysed by digestion with restriction enzymes to screen for desired clones.

Mutant construction

Site-specific mutagenesis was carried out by using the Oligonucleotide-Directed-*in-vitro*-Mutagenesis System of Amersham International according to the procedure recommended by the supplier, which was basically developed by Eckstein and co-workers (Taylor *et al.*, 1985). Single-stranded DNA was prepared from bacteriophage M13mp19 (rnh). Mutagenic primers used are summarized in Table 1. These oligodeoxynucleotides were synthesized by the phosphoramidite method (Beaucage & Caruthers, 1981) with an Applied Biosystems model 380A automatic synthesizer. Each oligodeoxynucleotide was purified by reverse-phase h.p.l.c. on a TSK-GEL ODS-120T (4.6 mm × 250 mm) column from Toyo-Soda (Tokyo, Japan) as described previously (Inoue *et al.*, 1987). Mutations were confirmed by DNA sequencing with the use of the chain-termination method of Sanger *et al.* (1977). Because the oligodeoxynucleotides directing site-specific mutations were usually designed to create a new restriction site in the gene, mutants were initially screened by restriction-enzyme mapping on replication-form DNA prepared from several plaques. Subsequently each *rnh* mutant was subcloned into plasmid pDR600 by insertion of the M13 bacteriophage *Xba*I–*Sst*II restriction fragments into the *Xba*I and *Sst*II sites of plasmid pDR600. The *Sst*II site, which is located about 400 bp downstream of the initial methionine codon of the *rnh* gene, is unique in both plasmid pDR600 and M13 bacteriophage containing the *rnh* gene. For construction of the mutant *rnh* genes, in which any two of three cysteine codons were replaced with that of alanine or serine, two mutagenic primers were mixed together and used for the site-directed mutagenesis. The plasmid vector for the cysteine-free enzyme was constructed by exchanging the DNA fragment between a plasmid that has the mutation at the Cys-13 codon and one that has mutations at both Cys-63 and Cys-133 codons. The unique restriction site of *Eco*47III, which is located between the Cys-13 and Cys-63 codons, was used for this purpose.

Overproduction and purification

An overproducing strain for each mutant RNAase H was constructed by transforming strain AK101 with one of the mutant plasmids. Cells were grown in Luria broth medium containing ampicillin and chloramphenicol. When the absorbance at 550 nm of the culture reached about 0.8, isopropyl β-D-thiogalactopyranoside (1 mM) was added to the culture medium and cultivation was continued for an additional 4 h. Cells were then harvested by centrifugation and subjected to purification procedures as described previously (Kanaya *et al.*, 1989). The purity of the enzyme was analysed by SDS/PAGE (Laemmli, 1970).

Assay for RNAase H activity

The enzyme activities were determined as described previously (Dirksen & Crouch, 1981) by measuring the radioactivity of the acid-soluble digestion product from poly([^{32}P]rA)·poly(dT).

Table 1. Sequences of mutagenic primers used to replace cysteine codons with either serine or alanine codons at specified positions in the structural gene of *E coli* RNAase H

The codon for the replaced amino acid residue is denoted by dots. The mismatched position in each oligonucleotide is underlined. The positions of the newly introduced restriction sites are enclosed in boxes.

Codon	Amino acid change	Mutant	Mutagenic primer	Restriction site
13	Cys→Ser	C13S	CCTGGATTGCCTAGGGACGAACCATCGG	*Sty*I
13	Cys→Ala	C13A	CCTGGATTGCCTAGGGCCGAACCATCGG	*Sty*I
63	Cys→Ser	C63S	GACTTCGCTATGTTCTTTTAACG	
63	Cys→Ala	C63A	GACTTCGGCATGTTCTTTTAACGC	
133	Cys→Ser	C133S	GGCACGAGCGAGCTCATCAGAGCGTTCG	*Sst*I
133	Cys→Ala	C133A	GGCACGAGCGAGCTCATCAGCGCGTTCG	*Sst*I

The specific activity is defined as units of enzyme activity (nmol of acid-soluble material produced in 15 min)/mg of protein. Protein concentrations of the mutant enzymes were determined from the u.v. absorption assuming that all mutant enzymes have the same absorption coefficient as that of the wild-type enzyme. It has previously been reported that the wild-type enzyme had an $A_{280}^{0.1\%}$ value of 1.5 (Kanaya *et al.*, 1989). Because 28.1 nmol of protein per A_{280} unit of the enzyme was recovered by amino acid analysis [corresponding to 0.494 mg of protein with M_r 17559 (Kanaya & Crouch, 1983)], we now use an $A_{280}^{0.1\%}$ value of 2.02. This corrected value of 2.02 compares reasonably well with the $A_{280}^{0.1\%}$ value of 2.24 calculated by using ϵ 1576 $M^{-1} \cdot cm^{-1}$ for tyrosine ($\times 5$) and ϵ 5225 $M^{-1} \cdot cm^{-1}$ for tryptophan ($\times 6$) at 280 nm (Goodwin & Morton, 1946).

Chemical modification with thiol-blocking reagents

A fresh solution of 0.2 M-DTNB in 0.05 M-sodium phosphate buffer, pH 7.1, 2 M-NEM in ethanol or 1.5 M-iodoacetic acid in 0.1 M-NaOH was prepared just before the reaction was started. The enzyme was dissolved in 0.1 M-Tris/HCl buffer, pH 8.0, at a concentration of 5–10 μM. This buffer was chosen for the reaction because the enzyme has an optimum pH of 8 for hydrolysis of the substrate and the thiol group must be appreciably reactive with thiol-blocking reagent at this pH. The reaction was started by addition of $\frac{1}{20}$ vol. of DTNB solution (final concn. 10 mM), $\frac{1}{100}$ vol. of NEM solution (final concn. 20 mM) or $\frac{1}{50}$ vol. of iodoacetic acid solution (final concn. 30 mM) to the enzyme solution and continued for 15 min at room temperature with gentle shaking. Then the reaction mixture was passed through a PD-10 column (1.4 cm × 5 cm) equilibrated with 10 mM-Tris/HCl buffer, pH 7.5, to remove excess of the reagent. In a control reaction the thiol-blocking reagent was omitted. Titration of thiol groups with DTNB was carried out according to the method previously reported (Ellman, 1959). The concentration of 5-mercapto-2-nitrobenzoate anion was determined from the molar absorption coefficient value of 14000 $M^{-1} \cdot cm^{-1}$ at 412 nm.

Characterization of primary structure

All mutated and chemically modified enzymes were digested at 37 °C and pH 9.0 (0.1 M-Tris/HCl buffer) for 30 min with lysyl endopeptidase at an enzyme/substrate ratio of 1:50 (by wt.). Chymotryptic digestion was carried out at 37 °C for 4 h in 0.1 M-Tris/HCl buffer, pH 8.0, with an enzyme/substrate ratio of 1:20 (by wt.). The resultant peptides were separated by reverse-phase h.p.l.c. on an Aquapore RP-300 column (4.6 mm × 250 mm) from Brownlee Laboratories (Santa Clara, CA, U.S.A.) as described previously (Kanaya & Uchida, 1986). Fractions containing the peptides were collected and analysed for amino acid sequence. Amino acid sequence analysis was performed by stepwise Edman degradation (Edman & Begg, 1967) in a gas-phase automated sequencer (Applied Biosystems model 477A) equipped with an on-line h.p.l.c. apparatus (Applied Biosystems model 120A). Amino acid analysis was carried out with a Beckman System 6300E automatic amino acid analyser. Samples were hydrolysed at 150 °C for 1.5 h by using a vapour-phase hydrolysis technique with constant-boiling HCl containing 0.5% (v/v) phenol.

Measurement of c.d. spectra

The c.d. spectra were measured on a J-600 automatic spectropolarimeter from Japan Spectroscopic Co. (Tokyo, Japan). Spectra were obtained with solutions containing enzyme at 0.25 mg/ml in 0.1 M-NaCl/10 mM-sodium acetate buffer, pH 5.5, for the mutant enzymes and at 0.1 mg/ml in 10 mM-Tris/HCl buffer, pH 7.5, for the modified enzymes in a cell with an optical path of 2 mm. The mean residue ellipticity, $[\theta]_{m.r.w.}$, has the unit degree $\cdot cm^2 \cdot dmol^{-1}$.

RESULTS

Peptide mapping of *E. coli* RNAase H

To facilitate the identification of the amino acid residues that were modified with chemical reagents or replaced by site-directed mutagenesis, the elution profile of the digestion products of the wild-type enzyme with lysyl endopeptidase on reverse-phase h.p.l.c. was examined (Fig. 1*b*). Lysyl endopeptidase is known to cleave specifically the peptide bond *C*-terminal of the lysine residue. This technique (peptide mapping) is useful to judge which peptide is unusual in the mutant or modified enzyme. Amino acid sequence analysis allowed us to identify all lysyl endopeptidase-digest peptides except LEP-1, LEP-6 and LEP-7 (Fig. 1*a*). These three peptides must be eluted in the flow-through

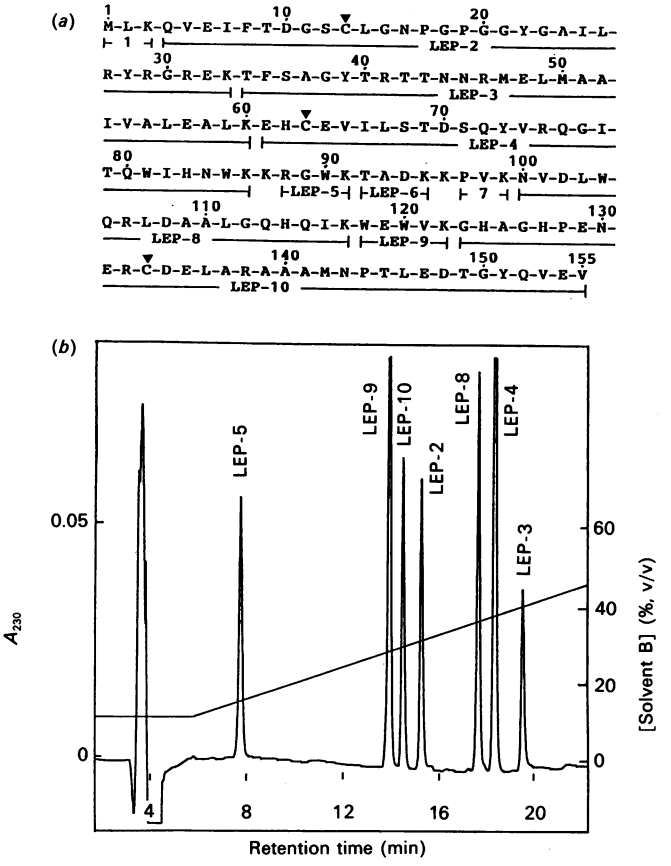

Fig. 1. Peptide-mapping analysis of *E. coli* RNAase H

(*a*) The amino acid sequence of *E. coli* RNAase H is shown. Peptides generated by digestion with lysyl endopeptidase are indicated and designated LEP followed by an arabic number indicating the order of the peptide alignment from the *N*-terminus in the protein molecule. (*b*) Separation of lysyl endopeptidase peptides of *E. coli* RNAase H on reverse-phase h.p.l.c. A lysyl endopeptidase digest (1 nmol) of *E. coli* RNAase H was directly loaded on an Aquapore RP-300 reverse-phase column (4.6 mm × 250 mm) equilibrated with 10% (v/v) solvent B in solvent A. Elution was performed by raising linearly the concentration of solvent B in solvent A from 10% to 60% (v/v) over 25 min. Solvent A was aq. 0.1% (v/v) trifluoroacetic acid and solvent B was acetonitrile containing 0.05% (v/v) trifluoroacetic acid. The flow rate was 1.0 ml/min. All the peaks were collected and analysed for amino acid sequences. The positions of the lysyl endopeptidase peptides except LEP-1, LEP-6 and LEP-7 are shown. These three peptides must be eluted in the flow-through fraction. The straight line represents the concentration of solvent B.

fraction from the reverse-phase column used, probably because of their poor hydrophobicities. The sequence of peptide LEP-1 could be identified by subjecting undigested intact protein to amino acid sequence analysis. The sequence involving peptides LEP-6 and LEP-7 was determined by subjecting the chymotryptic-digest peptide (Lys-91–Trp-104) to amino acid sequence analysis. This latter peptide was also isolated by reverse-phase h.p.l.c. after chymotryptic digestion. Thus the predicted amino acid sequence of *E. coli* RNAase H was confirmed for the entire molecule. In addition, it was shown that no cysteine residue was involved in disulphide bridge formation, because all three lysyl endopeptidase-digest peptides that contain a cysteine residue were separated by reverse-phase h.p.l.c.

Construction and overproduction of mutant enzymes

E. coli RNAases H with all possible combinations of cysteine → alanine and cysteine → serine substitutions were constructed by site-directed mutagenesis. The reason why alanine and serine were chosen to be replacements for cysteine was that conformational change due to the substitution was assumed to be minimized because of the similarities in the spatial volumes of their side chains. Because the enzyme molecule contains three cysteine residues, 14 mutant enzymes in total (seven for cysteine → alanine substitution and seven for cysteine → serine substitution) were generated. They are C13A, C63A, C133A, C13/63A, C13/133A, C63/133A and C13/63/133A for cysteine → alanine substitution, and C13S and so on for cysteine → serine substitution. The identity of each mutant enzyme was confirmed by determining the complete nucleotide sequence of the mutant gene or amino acid sequence of the mutant protein. All of these mutant enzymes were successfully overproduced in the *rnh*-deficient strain AK101.

The level of production in cells was estimated from the intensity of the band detected by Coomassie Brilliant Blue staining after SDS/PAGE of whole cell extract. It varied from 2 to 8 mg/l for the different mutant enzymes (3–4 mg/l for the wild-type enzyme). Clear relationships between the level of production and the position of the mutation were observed. Namely, replacement of Cys-63 by serine or alanine increased the level of production 2-fold. In contrast, replacement of Cys-133 with serine decreased it to one-half compared with the wild-type enzyme. Comparison of the amount of the enzyme in the soluble fraction after lysis by sonication with that in the whole-cell extract, by SDS/PAGE followed with Coomassie Brilliant Blue staining, indicated that all mutant enzymes except a series of mutant enzymes in which Cys-133 was replaced by serine accumulated in cells in a soluble form. The mutant enzymes C133S, C13/133S, C63/133S and C13/63/133S could be partially recovered in a soluble form, but the remainder (50–70%) was found in an insoluble fraction. All mutant enzymes were purified so as to give a single band on SDS/PAGE (Fig. 2). The yield from the crude lysate was about 80% in all cases.

Enzymic activity of mutant enzymes

Specific activities of the mutant enzymes were determined and are summarized in Table 2. Because any replacement of a cysteine residue by alanine as well as of Cys-63 by serine had almost no effect on the specific activity of the enzyme, it is obvious that none of the cysteine residues is required for activity. In addition, it was shown that a mutant enzyme in which all cysteine residues were replaced by alanine exhibited a specific activity similar to that of the wild-type enzyme. Thus none of the cysteine residues is indispensable for activity.

It has been previously reported that a reducing reagent is required for maximal activity (Berkower *et al.*, 1973). To our surprise, however, no decrease in enzyme activity was observed for the wild-type enzyme when the reducing reagent was omitted. The reason for this discrepancy remains unclear. The observation that a reducing reagent is not required for the enzyme activity agrees with our finding that no cysteine residue is involved in the active site. Although replacement of any cysteine residue by alanine did not greatly affect the activity, replacement of a cysteine residue by serine resulted in a relatively large change in the specific activity of the enzyme. The mutants C13S and C13/63S had twice as high a specific activity as did the wild-type enzyme, whereas the mutants C133S and C63/133S exhibited 50% of the specific activity of the wild-type enzyme. The mutant C13/63/133S had a specific activity similar to that of the wild-type enzyme, probably because the effect of each replacement on the enzyme activity was cumulative.

DTNB titration

DTNB titrations of the wild-type and three mutant enzymes with a single cysteine residue (C13/63A, C13/133A and C63/133A) were examined in the presence and in the absence of

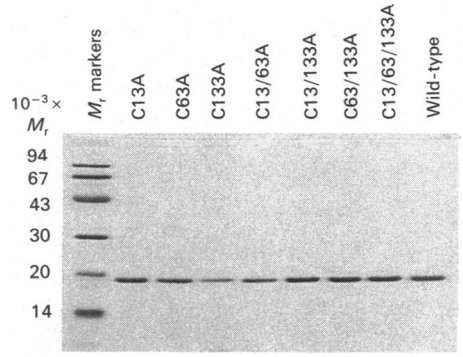

Fig. 2. SDS/PAGE of purified mutant *E. coli* RNAases H

Samples (1 μg) of mutant protein were subjected to electrophoresis on a 15% polyacrylamide gel and then stained with Coomassie Brilliant Blue. A low-M_r marker kit (Pharmacia) containing phosphorylase *b*, BSA, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin was used. All cysteine → alanine and cysteine → serine mutant enzymes were examined by two separate gels and only the gel for cysteine → alanine mutants is shown. All cysteine → serine mutants also gave a single band with equal mobility.

Table 2. Comparison of the specific activities of mutant *E. coli* RNAases H

Specific activities of all mutant enzymes were determined with poly([^{32}P]rA)·poly(dT) as a substrate. Those for the mutant enzymes in which two cysteine residues were replaced with alanine or serine are not shown, because they could be accounted for by the cumulative effect of individual cysteine replacements on the enzyme activity. Specific activities are expressed as means + S.E.M. ($n = 3$).

Mutant	Specific activity (units/mg)	Specific activity (% of wild-type)
Wild-type	$(24\pm3)\times10^4$	100
C13S	$(45\pm5)\times10^4$	190
C13A	$(27\pm4)\times10^4$	110
C63S	$(19\pm3)\times10^4$	80
C63A	$(19\pm2)\times10^4$	80
C133S	$(12\pm1)\times10^4$	50
C133A	$(19\pm3)\times10^4$	80
C13/63/133S	$(23\pm4)\times10^4$	95
C13/63/133A	$(24\pm4)\times10^4$	100

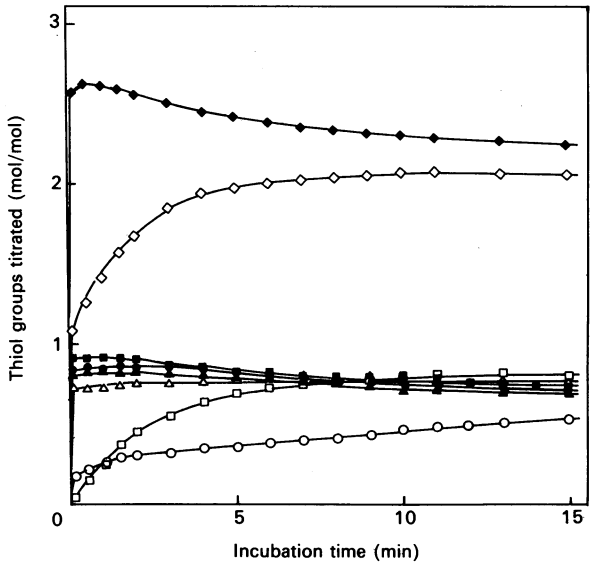

Fig. 3. DTNB titration of thiol groups of wild-type and mutant *E. coli* RNAases H

Wild-type and three mutant enzymes with a single cysteine residue at different positions were incubated at 0.125 mg/ml with 10 mM-DTNB in 0.1 M-Tris/HCl buffer, pH 8.0, at 25 °C. Enzymes: wild-type (◇ and ◆); C13/63A (○ and ●); C13/133A (△ and ▲); C63/133A (□ and ■). White and black symbols represent the results obtained in the absence and presence of 6 M-guanidinium chloride respectively.

6 M-guanidinium chloride, and the number of thiol groups titrated with DTNB was plotted against the time of reaction (Fig. 3). In the presence of 6 M-guanidinium chloride (denatured state), 2.7 and 0.9 mol of DTNB reacted with 1 mol of wild-type and mutant enzymes respectively. This indicates that every cysteine residue exists in an unmodified reduced form. In the denatured state the reaction of any cysteine residue with DTNB was rapid and was completed within 30 s. In contrast, distinct DTNB titrations were obtained for each of the three mutant enzymes in the absence of 6 M-guanidinium chloride (native state). Because the number of thiol groups titrated with DTNB in the wild-type enzyme was 2.1 mol/mol after 15 min, but was still gradually increasing because of the slow reaction of Cys-133 with DTNB, all cysteine residues seemed to be reactive with DTNB in the native state. Comparison of the DTNB titration in the native state with that of three mutant enzymes with a single cysteine residue allowed us to distinguish the behaviour of each individual cysteine residue. Cys-63 was the most reactive residue and its DTNB titration was completed within 30 s, suggesting that this residue is fully exposed to the solvent. Cys-13 reacted with DTNB rather slowly and it took several minutes for the reaction to be completed. Cys-133 was the most insensitive to the reaction with DTNB, and 30% of this residue remained unmodified even after 15 min. The titration of Cys-133 with DTNB could be completed if the reaction time was prolonged further. Because the DTNB titration curve of the wild-type enzyme in the native state was in good agreement with the adduct of those of the three mutant enzymes, the possibility that the environment of each cysteine residue was altered by the introduction of the mutation seems unlikely, but it cannot be completely excluded.

Chemical modification

To confirm the previous report that the enzyme is sensitive to NEM (Berkower *et al.*, 1973), the wild-type and three mutant enzymes with a single cysteine residue were treated with various thiol-blocking reagents. When the wild-type enzyme (0.1 mg/ml in 0.1 M-Tris/HCl buffer, pH 8.0) was treated with an excess of NEM or iodoacetic acid, white precipitates were observed in a few minutes. After gel filtration through PD-10, the modified enzyme was recovered in a soluble form with a yield of only a few per cent. No precipitate was observed when the mutant enzymes were treated with the same reagent. These modified mutant enzymes, as well as the wild-type enzyme that had been treated with DTNB, were recovered in a soluble form from PD-10 with a yield of nearly 100%. The reason for the decrease in solubility of the wild-type enzyme modified with NEM or iodoacetic acid remains unclear. The simultaneous modification of all cysteine residues with these reagents, which was confirmed by amino acid sequence analysis (results not shown), may cause a conformational change that results in the decrease of the solubility.

Table 3. Effect of the chemical modifications on the enzyme activities of wild-type and mutant *E. coli* RNAases H

Wild-type and mutant RNAases H modified with iodoacetic acid, NEM or DTNB were prepared as described in the Experimental section and subjected to determination of enzyme activity without separation from the unmodified native counterparts. Residual activity of each enzyme modified with various thiol-blocking reagents was determined by comparing its enzyme activity with that of the native counterpart. The enzyme activity of RNAase H is normally determined in the presence of 2-mercaptoethanol. However, for the assay of the enzyme modified with DTNB, 2-mercaptoethanol was omitted to avoid the regeneration of the native enzyme, because the modified group (5-mercapto-2-nitrobenzoate) was attached to a thiol group through a disulphide bond and could be removed by the addition of the reducing reagent. The amount of the protein was determined by the method of Bradford (1976), with wild-type enzyme as a standard.

		Residual activity (%)		
Mutant	Modifying agent...	Iodoacetic acid	NEM	DTNB
Wild-type		< 0.1	< 0.1	< 0.1
C13/63A		3.1	4.5	30
C13/133A		90	95	90
C63/133A		0.9	2.0	1.1
C13/63/133A		100	100	100

The residual activity of each modified enzyme was determined by comparing the specific activity of the modified enzyme with that of the unmodified enzyme, and the results are summarized in Table 3. The wild-type enzyme obviously lost enzyme activity almost completely as a result of treatment with these thiol-blocking reagents. When each mutant enzyme was treated with a thiol-blocking reagent, the C13/63A and C63/133A mutants lost activity, but the C13/133A mutant did not. Because DTNB titration experiments revealed that Cys-63 was exposed to solvent and was most reactive with DTNB, there is no doubt that this residue was modified. Thus the NEM-sensitivity of *E. coli* RNAase H was shown to be due to the alkylation of either Cys-13 or Cys-133. No significant differences in reactivities were observed for the thiol-blocking reagents examined, although there was an indication that DTNB was less reactive with Cys-133 than the other two reagents. The relatively high residual activity (30%) of the mutant C13/63A modified with DTNB therefore reflects that of the unmodified enzyme (Table 3).

Primary structures of the modified enzymes

The primary structure of each mutant enzyme (C13/63A, C13/133A and C63/133A) modified with NEM was further

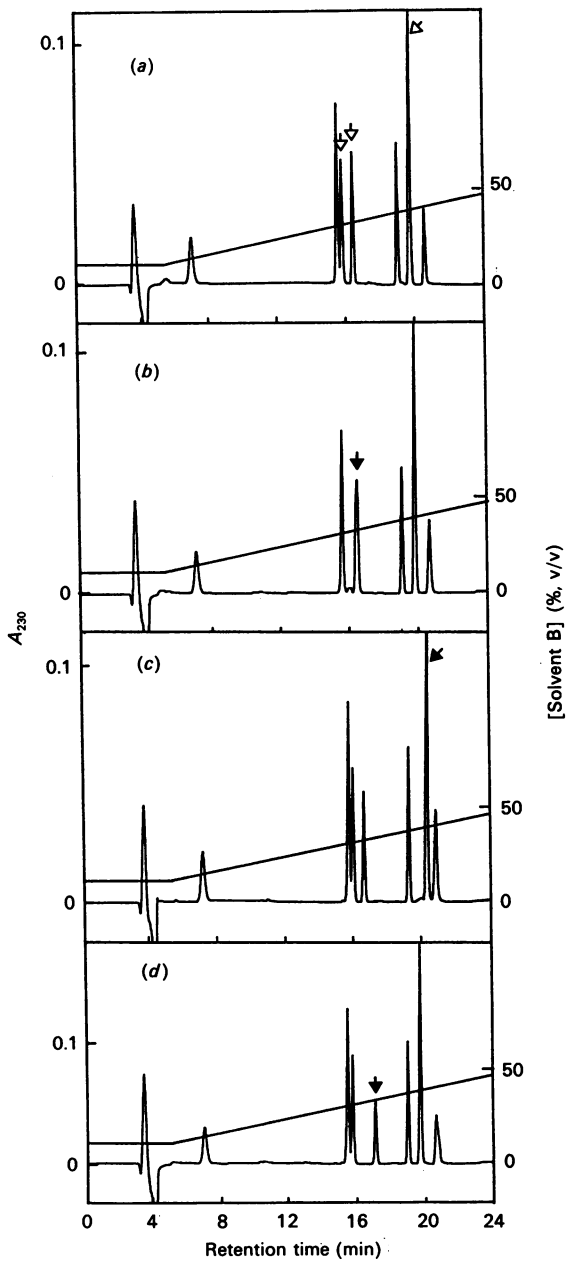

Fig. 4. Separation of lysyl endopeptidase digests of mutant *E. coli* RNAases H modified with NEM on reverse-phase h.p.l.c.

Lysyl endopeptidase digests (1 nmol) of mutant C13/63/133A (*a*), NEM-modified C13/63A (*b*), NEM-modified C13/133A (*c*) and NEM-modified C63/133A (*d*) were directly loaded on an Aquapore RP-300 reverse-phase column (4.6 mm × 250 mm) equilibrated with 10% (v/v) solvent B in solvent A. Elution was performed as described in the legend to Fig. 1. The positions of three peptides in which each cysteine residue was replaced with alanine are indicated by white arrows in (*a*). The positions of the peptides in which the cysteine residue was alkylated with NEM are indicated by black arrows. Notice that LEP-10 containing an NEM-modified cysteine residue was eluted almost together with LEP-2 in (*b*).

examined by means of peptide mapping analysis to confirm that cysteine was the only amino acid residue modified with NEM. The elution profile of the lysyl endopeptidase-digest peptides of each modified enzyme was compared with that of the cysteine-free mutant C13/63/133A (Fig. 4). It has already been shown which peptide has a free thiol group (Fig. 1). Only the position of the peptide that contains a cysteine residue was shifted by treatment with NEM (shown for each mutant enzyme as black arrows in Fig. 4). The altered elution of the modified peptides probably reflects the enhanced hydrophobicity of the peptide resulting from alkylation of the cysteine residue. The determination of the amino acid sequence of the altered peptide permitted the identification of the cysteine residue as the only amino acid residue modified by NEM. Likewise it was confirmed that no amino acid residues other than three cysteine residues were modified in the wild-type enzyme inactivated with NEM (results not shown). NEM–cysteine was identified by using L-cysteine treated with NEM. The phenylthiohydantoin derivative of NEM–cysteine was eluted between the proline phenylthiohydantoin derivative and the methionine phenylthiohydantoin derivative from the on-line h.p.l.c. column PTH C_{18} (2.1 mm × 220 mm). Although there is no structural evidence for the presence of *S*-(ethylsuccinimido)cysteine, which is the product of the reaction between cysteine and NEM, there is no doubt that the cysteine residue in the enzyme gave the same product as L-cysteine treated with NEM.

It is noteworthy that all lysyl endopeptidase-digest peptides in which cysteine was replaced by serine were eluted from the reverse-phase column at a lower acetonitrile concentration than those with a cysteine residue (results not shown). In contrast, those with cysteine → alanine substitutions were eluted at positions similar to those of the wild-type enzyme. Elution of these peptides thus reflected the difference in the hydrophobicity of the replaced amino acid residue (Kidera *et al.*, 1985).

C.d. spectra

C.d. spectra examined in the region from 200 nm to 260 nm for all mutant enzymes were similar to that of the wild-type enzyme (Fig. 5*a*). Furthermore, each mutant enzyme with a single cysteine residue modified with NEM gave a c.d. spectrum virtually identical with that of the unmodified counterpart (Fig. 5*b*). This result indicated that inactivation with NEM was not due to marked changes in the tertiary structure.

DISCUSSION

E. coli RNAase H contains three free cysteine residues. To clarify the role of these cysteine residues, they were replaced by other amino acid residues by site-directed mutagenesis, and the effects of the mutation on enzyme activity and on inactivation by thiol-blocking reagents were examined. As a result, it was found that none of the cysteine residues was essential for the enzyme activity, and that modification of either Cys-13 or Cys-133 resulted in the loss of the enzyme activity but modification of Cys-63 did not. Because c.d. spectra did not show any change in the tertiary structure of each modified mutant enzyme, it is likely that a steric defect at the local area introduced by the chemical modification led to inactivation of the enzyme. C.d. spectra in the far-u.v. region may fail to distinguish such a subtle change.

Others have reported that a cysteine residue, presumed to be essential from inhibition experiments with thiol-blocking reagents, was not required for enzyme activity for glycyl-tRNA synthetase from *E. coli* (Profy & Schimmel, 1986), glutathione synthetase from *E. coli* B (Kato *et al.*, 1988) and D-amino acid transaminase from *Bacillus sphaericus* (Merola *et al.*, 1989). It has been proposed in each case that the sensitivity of the enzyme to thiol-blocking reagents arises from steric or conformational effects of the alkylated cysteine side chain.

Replacement of Cys-13 by serine increased the specific activity to twice that of the wild-type enzyme. The reason for this remains unclear. It is noteworthy, however, that alignment of *E. coli* RNAase H sequence with various retroviral reverse transcriptase RNAases H made by computer analysis (Johnson

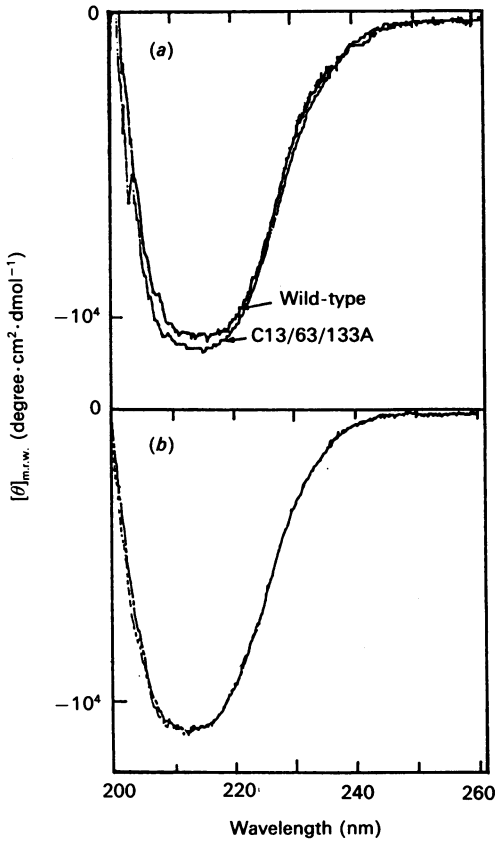

Fig. 5. Comparison of far-u.v. c.d. spectra of wild-type, mutant and NEM-modified mutant *E. coli* RNAases H

(*a*) All mutant enzymes were examined, and the spectrum of mutant C13/63/133A as a representative is shown in comparison with that of the wild-type enzyme. (*b*) Effect of the chemical modification with NEM on the c.d. spectrum was examined for three mutants with a single cysteine residue. Only the spectrum of NEM-modified C63/133A, as a representative, is shown in comparison with that of the unmodified C63/133A. All spectra were measured as described in the Experimental section.

et al., 1986) indicated that serine is a conserved residue at this position. This suggests the possibility that the serine residue can contribute to making a more effective enzyme. In contrast with the replacement of Cys-13 by serine, replacement of Cys-133 by serine resulted in a decrease in the specific activity of the enzyme. Because Cys-133 is relatively insensitive to DTNB, the side chain of Cys-133 seems to be located inside the molecule. Therefore it can be imagined that a replacement of cysteine by serine, which has much more hydrophilic side chain, caused some distortion in this region. When Cys-133 was replaced by alanine, the specific activity of the enzyme was not altered as much. This suggests that the hydrophobic interaction between Cys-133 and some other nearby amino acid residues may make some contribution to the maintenance of tertiary structure of the enzyme.

Because chemical modification of either Cys-13 or Cys-133 with a thiol-blocking reagent caused inactivation of the enzyme, these residues might be located close to the active site. Alterations in the specific activity by the replacement of these residues with serine also support this prediction. There is no doubt that Cys-63 is spatially located far from the active site of the molecule, because neither the replacement of Cys-63 by other amino acids nor the chemical modification with thiol-blocking reagents affected the enzyme activity. Similar relationships between the reactivity of the thiol group with DTNB and the effect of the modification on the enzyme activity have been reported for many other proteins (Wilson *et al.*, 1980), namely that external unhindered thiol functions can be titrated rapidly, often with no loss of enzyme activity. In many other cases the slower reaction of less readily accessible thiol groups is coupled with loss of the activity.

According to the main chain folding of *E. coli* RNAase H, which has recently been determined by X-ray analysis (Katayanagi *et al.*, 1990), Cys-13 and Cys-63 are exposed to the solvent, although the former seems to be less exposed because of the obstruction by an adjacent amino acid, whereas Cys-133 is situated in a side of α-helix facing toward two β-strands, i.e. a thiol group of Cys-133 is in a sterically restricted environment that is hydrophobic in character. Thus the decrease in susceptibility to reaction with DTNB of Cys-63 > Cys-13 > Cys-133 is in good agreement with the localization of the cysteine residues in the protein structure. In addition, Cys-13 and Cys-133 are situated relatively close to the putative Mg^{2+}-binding site, which must be a part of the active site of *E. coli* RNAase H. It is therefore likely that introduction of a bulky side chain on to either of these cysteine residues by chemical modification forces an altered disposition of the active-site residues. Such subtle shifts of the position of the active-site residues may be detrimental for activity. Another possibility that also cannot be ruled out is that the bulky group incorporated by chemical modification may interfere with the binding of the substrate to the active site of the enzyme. Refinement of the three-dimensional structure of *E. coli* RNAase H, as well as that of the mutant enzymes, will permit resolution of the question concerning why chemical modification of either Cys-13 or Cys-133 causes inactivation and why replacement of either of these residues by serine alters the specific activity of the enzyme.

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