Human RNase H1 Activity Is Regulated by a Unique Redox Switch Formed between Adjacent Cysteines

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Human RNase H1 is active only under reduced conditions. Oxidation as well as N-ethylmaleimide (NEM) treatment of human RNase H1 ablates the cleavage activity. The oxidized and NEM alkylated forms of human RNase H1 exhibited binding affinities for the heteroduplex substrate comparable with the reduced form of the enzyme. Mutants of human RNase H1 in which the cysteines were either deleted or substituted with alanine exhibited cleavage rates comparable with the reduced form of the enzyme, suggesting that the cysteine residues were not required for catalysis. The cysteine residues responsible for the observed redox-dependent activity of human RNase H1 were determined by site-directed mutagenesis to involve Cys147 and Cys148. The redox states of the Cys147 and Cys148 residues were determined by digesting the reduced, oxidized, and NEM-treated forms of human RNase H1 with trypsin and analyzing the cysteine containing tryptic fragments by high performance liquid chromatography-electrospray ionization-Fourier transform ion cyclotron mass spectrometry. The tryptic fragment Asp131–Arg153 containing Cys147 and Cys148 was identified. The mass spectra for the Asp131–Arg153 peptides from the oxidized and reduced forms of human RNase H1 in the presence and absence of NEM showed peptide masses consistent with the formation of a disulfide bond between Cys147 and Cys148. These data show that the formation of a disulfide bond between adjacent Cys147 and Cys148 residues results in an inactive enzyme conformation and provides further insights into the interaction between human RNase H1 and the heteroduplex substrate.

RNase H hydrolyzes RNA in RNA-DNA hybrids (1). RNase H activity appears to be ubiquitous in eukaryotes and bacteria (2–7). Although RNases H constitute a family of proteins of varying molecular weight, the nucleolytic activity and substrate requirements appear to be similar for the various isoforms. For example, all RNases H studied to date function as endonucleases exhibiting limited sequence specificity and requiring divalent cations (e.g. Mg2+, Mn2+) to produce cleavage products with 5’ phosphate and 3’-hydroxyl termini (8).

Two classes of RNase H enzymes have been identified in mammalian cells (5, 9, 10). These enzymes were shown to differ with respect to co-factor requirements and were shown to be inhibited by sulfhydryl reagents (10, 11). Although the biological roles of the mammalian enzymes are not fully understood, it has been suggested that mammalian RNase H1 may be involved in replication and that the RNase H2 enzyme may be involved in transcription (12, 13).

Recently, two human RNase H genes have been cloned and expressed (11, 14, 15). RNase H1 is a 286-amino acid protein and is expressed ubiquitously in human cells and tissues (11). The amino acid sequence of human RNase H1 displays strong homology with RNase H1 from yeast, chicken, Escherichia coli, and mouse (11). The human RNase H2 enzyme is a 299-amino acid protein with a calculated mass of 33.4 kDa and has also been shown to be ubiquitously expressed in human cells and tissues (14).

The properties of the cloned and expressed human RNase H1 have recently been characterized and many of the properties observed for human RNase H1 are consistent with the E. coli RNase H1 isotype, (e.g. the co-factor requirements, substrate specificity and binding specificity) (16, 17). In fact, the carboxyl-terminal portion of human RNase H1 is highly conserved with the amino acid sequence of the E. coli enzyme. The glutamic acid and two aspartic acid residues of the catalytic site, as well as the histidine and aspartic acid residues of the proposed second divalent cation binding site of the E. coli enzyme are conserved in human RNase H1 (18–21). In addition, the lysine residues within the highly basic α-helical substrate-binding region of E. coli RNase H1 are also conserved in the human enzyme. Site-directed mutagenesis of the catalytic amino acids and the basic residues of the substrate-binding domain of human RNase H1 showed that these conserved residues are required for activity (22).

Despite these similarities, the structures of the two enzymes differ in a number of important properties. For example, the amino acid sequence of human RNase H1 is ~2-fold longer than the E. coli enzyme. The human enzyme contains a 73 amino acid region homologous with the RNA-binding domain of yeast RNase H1 at the amino terminus of the protein, which is separated from the conserved E. coli RNase H1 region by a 62-amino acid spacer region (22–24). Mutants in which the RNA-binding domain and spacer region of human RNase H1 were deleted showed that the RNA-binding domain was not required for RNase H activity and that this region was responsible for the observed positional preference for cleavage displayed by the enzyme as well as the enhanced binding affinity of the enzyme for various polynucleotides (22). The spacer region, on the other hand, was required for RNase H activity as the deletion of this region resulted in a significant reduction in both kcat and Km for the enzyme.

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One biochemical property that has been used to classify RNase H enzymes is the sensitivity to sulphydryl alkylating reagents such as N-ethylmaleimide (NEM)\(^2\) (10, 11, 25, 26). In general, RNase H1 enzymes are inhibited by NEM and both the *E. coli* and human enzymes share this property. In the case of *E. coli* RNase H1, NEM was shown to alkylate all three cysteine residues of the enzyme, although it was determined that alklylation of Cys\(^{133}\) and Cys\(^{133}\) was essential for the observed loss in enzymatic activity (26). Furthermore, site-directed mutagenesis of the cysteine residues of *E. coli* RNase H1 showed that these residues were not required for endonuclease activity. Finally, the *E. coli* enzyme was shown to be active under both reduced and oxidized conditions (26). These results suggest that the cysteines are not involved in catalysis but are positioned such that alklylation of the cysteines sterically interferes with substrate binding.

Comparison of the amino acid sequence of Human RNase H1 with the *E. coli* enzyme indicates that of the five cysteine residues found in human RNase H1, only Cys\(^{148}\) is conserved (Table I). In fact, this cysteine residue is highly conserved in both prokaryotic and eukaryotic RNases H1. Human RNase H1 contains an additional cysteine adjacent to Cys\(^{148}\), and this residue is conserved in RNases H1 from vertebrates (Table I).

In this study, we have explored the role of the cysteine residues of Human RNase H1 with respect to the function of the enzyme. We have determined the optimum redox state for the protein as well as the effect of the redox state on the binding and catalytic properties of the enzyme. Finally, we have identified a unique redox switch formed by vicinal cysteine residues.

### MATERIALS AND METHODS

**Preparation of Human RNase H1**—Human RNase H1 was prepared and purified as previously described (11, 16). The oxidized form of the enzyme was prepared by resuspending the lyophilized protein in dilution buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 50% glycerol) to a final concentration of 0.5 mg/ml and incubating at 25 °C for 1–4 h. The non-reduced form of human RNase H1 was prepared by resuspending the lyophilized protein in dilution buffer and adding 20–50 mM β-mercaptoethanol (BME) or 0.1–1 mM triis(2-carboxyethyl)phosphatase (TCERP). Human RNase H1 was alkylated with NEM by reducing the protein with TCEP as described, adding 10–20 μM NEM and incubating for 1 h at 25 °C. The reduced and oxidized forms of the enzyme were analyzed by SDS-PAGE.

**Construction of Cysteine Mutants**—The mutagenesis of human RNase H1 was performed using a PCR-based technique derived from Landt et al. (27). Briefly, two separate PCR were performed using sets of site-directed mutagenic primers and two vector-specific primers (11). For the RNase H1(C147A148) mutant, the 5′-oligodeoxynucleotide used for PCR was CTGATGCCTGGGTTAA GTAGCAGCGC, and the 3′-oligodeoxynucleotide was TTATCGAAGACCGCCACTATGTG TACCCGAG. The primers for RNase H1(C147A148) were 5′-CTG ATGGCGCTGGCAGTATGAGGCGTAA and 3′-TATCTTGGACCG CCAGGGTGACGAG. The primers for RNase H1(C148A) were 5′-CTGATGGCTGCGCTTCAATGGGGCTGA and 3′-TATCTGGAAGCAGCAGCACTATGGGCGTAA and 3′-TATCTGGAAGCAGCAGCACTATGGGCGTAA.

**Synthesis of Oligonucleotides**—The oligonucleotides were synthesized on a PE-ABI 380B synthesizer using 5′-O-ethyl-2′-O-bis(2-acetamido)-2-methylribonucleoside phosphoramidites and procedures described elsewhere (29). The oligonucleotides were purified by reverse phase HPLC. The DNA oligonucleotides were synthesized on a PE-ABI 380B automated DNA synthesizer and standard phosphoramidite chemistry. The DNA oligonucleotides were purified by precipitation two times out of 0.5 M NaCl with 2.5 volumes of ethyl alcohol.

**Preparation of 32P-Labeled Substrate**—The RNA substrate was 5′-end-labeled with \(^{32}P\) using 20 μl of T4 polynucleotide kinase (Promega), 120 pmol (7000 Ci/mmol) of \(\gamma^{32}P\)-ATP (ICN), 40 pmol of RNA, 70 mM Tris, pH 7.6, 10 mM MgCl\(_2\), and 50 mM dithiothreitol. The kinase reaction was incubated at 37 °C for 30 min. The labeled oligonucleotide was purified by electrophoresis on a 12% denaturing polyacrylamide gel (30). The specific activity of the labeled oligonucleotide is ~3000–8000 cpm/nmol.

**Preparation of the Heteroduplex**—The heteroduplex substrates were prepared in 100 μl containing 50 mM unlabeled oligoribonucleotide, 10 mM \(^{32}P\)-labeled oligodeoxynucleotide, 100 mM complementary oligodeoxynucleotide, and hybridization buffer (20 mM Tris, pH 7.5, 20 mM KCl). Reactions were heated at 90 °C for 5 min, cooled to 37 °C, and 60 units of Prime RNase Inhibitor (5 Prime → 3 Prime, Boulder, CO) and MgCl\(_2\) at a final concentration of 1 mM were added. Hybridization reactions were incubated 2–16 h at 37 °C, and BME was added at final concentration ranging from 0 to 200 mM.

**Determination of Initial Rates**—The heteroduplex substrates were digested with 0.5 ng of human RNase H1 at 37 °C. A 10-μl aliquot of the cleavage reaction was removed at time points ranging from 0 to 120 min and quenched by adding 5 μl of stop solution (8 μl urea and 500 mM EDTTA). The aliquots were heated at 90 °C for 2 min, resolved in a 12% denaturing polyacrylamide gel, and the subunit and product bands were quantitated on a Amersham Biosciences PhosphorImager. The quantitation of the converted product was plotted as a function of time. The initial cleavage rate was obtained from the slope (mole of RNA cleaved per minute) of the best-fit line for the linear portion of the plot, which comprises, in general, <10% of the total reaction and data from at least five time points.

**Competition experiments** were performed as described for the determination of initial rates with the exception that the hybridization reactions were prepared with 200 μM oligodeoxynucleotide, 10 μM oligoribonucleotide, and hybridization buffer without BME. Oxidized human RNase H1 was added to the hybridization reaction at final concentrations of 0.5 and 2.5 ng of protein. Alternatively, 20 μM BME and
NEM-alkylated enzyme was added to the hybridization reaction at final concentrations of 0.5 and 2.5 ng. The hybridization reactions were digested with 250 pg of the reduced form of human RNase H1. The reactions were quenched, analyzed, and quantitated as described for the determination of initial rates.

**Gel Renaturation Assay**—The gel renaturation assay was performed as described previously (6). Briefly, a 12% SDS-polyacrylamide gel containing 300,000 cpn of [32P]-labeled poly(A)poly(U) per 13 cm gel was prepared. Following electrophoresis the SDS was removed by washing the gel with three changes of 50 mM Tris, pH 8.0, 1 mol BME, 0.1 mol EDTA, and 25% (v/v) isopropanol for 5 min at 25 °C. The isopropanol was removed by washing the gel with two changes of 10 mM Tris, pH 8.0, and 5 mol BME for 15 min at 25 °C. The proteins were denatured by soaking the gel for 2 h at 25 °C with 50 mol Tris, pH 8.0, 20 mol BME, 10 mol MgCl₂, 50 mol NaCl, 6 mol guanidine HCl, and 10% (v/v) glycerol. The proteins were renatured by washing the gel with three changes of 50 mol Tris, pH 8.0, 20 mol BME, 10 mol MgCl₂, 50 mol NaCl, 2.5% Nonidet P-40, and 10% (v/v) glycerol for 20 h at 25 °C for reduced conditions and without BME for renaturation under oxidized conditions. Soluble radioactive was washed from the gel with four changes of 5% (v/v) trichloroacetic acid and 1% (v/v) sodium pyrophosphate for 15 min at 25 °C. The gel was quantitated on a PhosphorImager.

**Trypsin Digestion and Mass Spectral Analysis of Human RNase H1 Proteins**—Trypsin digestion of human RNase H1 proteins was prepared in 30 μl containing 2 μg human RNase H1, 0.87 M urea, 50 mol Tris-HCl, and 0.9 mol CaCl₂ and a (trypsin:RNase H1) ratio of 1:75 (w/w). Digestion reactions were incubated for 2 h at 65 °C. Immediately after removing the samples from the hot water bath, 3 μl of Me₂SO was added to the mixture to enhance the solubility of hydrophobic peptides.

Samples with the reducing agent were prepared as above except with the addition of 5 mM TCEP. The reaction was allowed to proceed at room temperature for 1 h. In selected experiments, NEM (10 mM final concentration, shaken at room temperature for 3 h) was introduced at this point to irreversibly "cap" the free sulfhydryl groups before adding trypsin.

**μHPLC-ESI-FTICR Mass Spectrometry**—A Zorbax C18 0.32 × 150-mm capillary silica column (Micro-Tech Scientific, Sunnyvale, CA) was employed on a Micro-Tech Ultra-Plus II HPLC system and directly coupled to the mass spectrometer. The mobile phases were 1% formic acid, 10% Me₆SO (mobile phase A), and 1% formic acid, 10% Me₆SO in acetonitrile (mobile phase B). Samples containing 25 μl of the human RNase H1 tryptic digest solution were injected onto the HPLC column, equilibrated with 99% A and 1% B at 4 μl/min, and eluted with 99% B and 1% A, also at 4 μl/min.

Experiments were performed on a modified Bruker Daltonics (Billerica, MA) Apex II 94e electrospray ionization-Fourier transform ion cyclotron (ESI-FTICR) mass spectrometer (35) with an actively shielded 9.4-tesla superconducting magnet. μHPLC-ESI-FTICR mass spectra were acquired at 6-s intervals and subsequently processed using the ICR2LS software package (Pacific Northwest National Laboratory, Richmond, WA).

**RESULTS**

The enzymatic activity of human RNase H1 under oxidized and reduced conditions is shown in Table IIA. Oxidation of human RNase H1 resulted in the ablation of cleavage activity. The initial cleavage rate (V₀) observed for the enzyme under reduced conditions was greater than 3 orders of magnitude faster than the rate observed for the oxidized enzyme. The loss of the enzymatic activity resulting from the oxidation of human RNase H1 was observed to be reversible (Table IIA). The enzymatic activity for Human RNase H1 was regenerated to the level of activity observed for the reduced form when the oxidized enzyme was incubated with 20 μM BME for 10 min. Furthermore, the enzyme activity was rapidly regenerated without requiring gradual reduction of the protein through gradient methods such as dialysis suggesting that regeneration of the enzyme was rapid and cooperative. The initial cleavage rate for human RNase H1 increased as a function of the concentration of the reducing agent BME (Fig. 1). The enzyme was most active at BME concentrations = 20 μM, and no loss in enzymatic activity was observed at 200 μM BME. Finally, analysis of the oxidized and reduced forms of human RNase H1 by SDS-PAGE showed that both forms migrated as monomers on the gel (data not shown).

The oxidized form of human RNase H1 was observed to competitively inhibit the endoribonuclease activity of the reduced form of the enzyme (Table IIB). These experiments were performed under single-turnover kinetics with the concentration of the oxidized form of the enzyme in excess of the reduced enzyme concentration. The V₀ for the reduced form of the enzyme was 2-fold faster than the cleavage rate observed for the reduced form of the enzyme in the presence of 2-fold excess oxidized human RNase H1. In the presence of 10-fold excess oxidized enzyme, the initial cleavage rate for the reduced form of human RNase H1 was below the detection limit of the assay. Initial cleavage rates were also determined under multiple-turnover kinetics with the substrate concentration in excess of the enzyme concentration and with the concentration of the oxidized enzyme in 10-fold excess over the reduced form of human RNase H1. Competition experiments under multiple-turnover conditions showed no reduction in the cleavage rate compared with the reduced form of human RNase H1 in the absence of oxidized enzyme (data not shown).

Competition experiments were also performed with NEM-alkylated human RNase H1. Here, the concentration of the reduced form of human RNase H1 was in excess of the substrate concentration, and the concentration of the NEM-alkylated protein was in excess of the reduced enzyme concentration. The V₀ for the reduced form of the enzyme was greater

| A: Redox state | V₀ (pM min⁻¹) |
|---------------|--------------|
| Oxidized RNase H1 | <Detectable limit |
| Reduced RNase H1 | 3500 ± 50 |
| Oxidized → reduced RNase H1 | 3328 ± 104 |

| B: Ratio (oxidized:reduced) | V₀ (pM min⁻¹) |
|---------------------------|--------------|
| (0:1) | 7150 ± 250 |
| (2:1) | 3825 ± 325 |
| (10:1) | <Detectable limit |

| C: Ratio (NEM labeled:reduced) | V₀ (pM min⁻¹) |
|-------------------------------|--------------|
| (0:1) | 9200 |
| (2:1) | 2500 |
| (10:1) | <Detectable limit |

* The detection limit of the assay corresponds to ~1% of the heteroduplex substrate cleaved over 60 min.

**TABLE II**

| V₀, for the reduced and oxidized forms of human RNase H1 |
|---------------------------------------------------------|
| A: initial rate measurements for human RNase H1 under oxidized and reduced conditions was determined as described under "Materials and Methods." The V₀ values are an average of three measurements with estimated errors of the coefficient of variation <10%. B: competition experiments were performed as described under "Materials and Methods." The heteroduplex substrate was incubated with the oxidized form of human RNase H1 prior to adding the reduced form of the enzyme. The concentration of the reduced form of human RNase H1 enzyme was in excess of the substrate concentration. The concentration of the oxidized form of human RNase H1 was 10-fold in excess of the reduced enzyme. The initial rate for the reduced form of human RNase H1 enzyme alone and in the presence of the oxidized form of the enzyme determined as described under "Materials and Methods." C: the competition experiments were performed as described in B except that excess NEM-labeled human RNase H1 was used as the competing protein.

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ranging from 0 to 200 mM as described under "Material and Methods." The Vₘ values are an average of three measurements with estimated errors of CV < 10%.

To identify the cysteine residues responsible for the observed loss the cleavage activity under oxidized conditions, the initial cleavage rate for the human RNase H1 deletion mutant H₁[Δ1–73] was determined. This deletion mutant was missing the first 73 amino acids from the NH₂ terminus of the protein and therefore lacked the C18 and C48 residues (Fig. 2A). The cleavage rate for the H₁[Δ1–73] mutant was compared with the wild-type human RNase H1 enzyme in the presence of increasing BME concentration (Fig. 1). Similar to the wild-type enzyme, the H₁[Δ1–73] mutant was inactive under oxidized conditions, and the cleavage rate of the H₁[Δ1–73] mutant increased with increasing concentration of BME.

Four mutants of human RNase H1 were prepared in which the three remaining cysteines were substituted with alanine (e.g. C147A, C148A, C191A, and C147A/C148A) (Fig. 2A). The cleavage activity of these mutants under both reduced and oxidized conditions was determined using a gel renaturation assay (Fig. 3). Here the enzymes are separated under denaturing conditions on an SDS-polyacrylamide gel containing 32P-labeled heteroduplex substrate as a result of human RNase H1 digestion.

The cleavage activity of the C₁₄₇A, C₁₄₈A, C₁₉₁A, and C₁₄₇A/C₁₄₈A mutants of human RNase H1 was determined by gel renaturation assay as described under "Materials and Methods." The mutants were renatured in the presence of 20 mM BME (reduced) and absence of BME (oxidized). The white bands correspond to the absence of 32P-labeled heteroduplex substrate as a result of human RNase H1 digestion.

A tryptic fragment containing the Cys₁⁴⁷⁻Cys₁⁴⁸ residues was identified for the oxidized- and reduced-ENEM-, and oxidized-ENEM-treated human RNase H1 (Fig. 2B). Analysis of the mass spectrometry scans for the fragment Asp₁³¹⁻Arg₁⁵³ generated under oxidized conditions revealed a signal between scans 100 and 114 corresponding to a mass of 2519.999 ± 0.006 Da, which was consistent with the calculated mass of 2520.003 Da for this fragment minus two hydrogen atoms and was consistent with the two cystines forming a disulfide bond (Fig. 4a). No signal was observed for the reduced form of the protein (Fig. 4a). A search of the mass spectrometry scans for the Asp₁³¹⁻Arg₁⁵³ fragment from the reduced/ENEM-treated enzyme revealed a signal corresponding to a mass of 2772.118 ± 0.006 Da (Fig.
Human RNase H1 was shown to be active only under reduced conditions. Oxidation of human RNase H1 resulted in the ablation of the cleavage activity (Table IIA). The cleavage rate increased with increasing BME concentrations and in fact human RNase H1 was observed to be most active under fully reduced conditions (e.g. BME concentrations as high as 200 mM), suggesting that the active conformation of the enzyme does not contain disulfide bonds (Fig. 1). Analysis of the oxidized form of the enzyme by SDS-polyacrylamide gel electrophoresis indicated a single protein with a molecular weight consistent with the calculated mass for the peptide minus two hydrogen atoms and no signal for either the reduced- or double NEM-labeled fragments (Fig. 4c).

**DISCUSSION**

Human RNase H1 was shown to be active only under reduced conditions. Oxidation of human RNase H1 resulted in the ablation of the cleavage activity (Table IIA). The cleavage rate increased with increasing BME concentrations and in fact human RNase H1 was observed to be most active under fully reduced conditions (e.g. BME concentrations as high as 200 mM), suggesting that the active conformation of the enzyme does not contain disulfide bonds (Fig. 1). Analysis of the oxidized form of the enzyme by SDS-polyacrylamide gel electrophoresis indicated a single protein with a molecular weight consistent with the calculated mass for the peptide minus two hydrogen atoms and no signal for either the reduced- or double NEM-labeled fragments (Fig. 4c).

4b). The observed mass was in excellent agreement with the calculated mass of 2772.114 Da for a double NEM-labeled Asp131-Arg153 fragment. Again, no signal was observed for either the oxidized or reduced forms of the Asp131-Arg153 fragment (Fig. 4b). Finally, analysis of the mass spectrometry scans for the Asp131-Arg153 fragment from the oxidized/NEM-treated RNase H1 showed a signal corresponding to a mass consistent with the calculated mass for the peptide minus two hydrogen atoms and no signal for either the reduced- or double NEM-labeled fragments (Fig. 4c).

**Fig. 4.** A series of composite selected mass chromatogram of the tryptic fragment containing Cys147 and Cys148. Human RNase H1 was digested with trypsin and the tryptic peptides identified by μHPLC-ESI-FITCR mass spectrometry as described under “Materials and Methods.” a, trypsin digestion of the oxidized form of human RNase H1. b, trypsin digestion of the reduced form of human RNase H1 labeled with NEM. c, trypsin digestion of the oxidized form of human RNase H1 treated with NEM. Each scan was searched for: the oxidized form of human RNase H1 (C) (RSSR), reduced form (-) (RSH, HSR), and double NEM-labeled enzyme (RS-NEM, NEM-SR).

**Discussion**

Human RNase H1 was shown to be active only under reduced conditions. Oxidation of human RNase H1 resulted in the ablation of the cleavage activity (Table IIA). The cleavage rate increased with increasing BME concentrations and in fact human RNase H1 was observed to be most active under fully reduced conditions (e.g. BME concentrations as high as 200 mM), suggesting that the active conformation of the enzyme does not contain disulfide bonds (Fig. 1). Analysis of the oxidized form of the enzyme by SDS-polyacrylamide gel electrophoresis indicated a single protein with a molecular weight consistent with the calculated mass for the peptide minus two hydrogen atoms and no signal for either the reduced- or double NEM-labeled fragments (Fig. 4c).
gesting that similar to E. coli RNase H1, the cysteine residues of human RNase H1 are not required for cleavage activity (26).

The redox states of the Cys\textsuperscript{147} and Cys\textsuperscript{148} residues for the oxidized and reduced forms of human RNase H1 were determined by treating the enzymes with trypsin and analyzing the cysteine containing tryptic fragments by μHPLC-ESI-FTICR mass spectrometry (32, 33). The oxidized form of human RNase H1 was also treated with NEM (oxidized/NEM) prior to trypsin digestion to eliminate the possibility of potential disulfide bonds between tryptic fragments. A tryptic fragment (Asp\textsuperscript{131}–Arg\textsuperscript{147}) containing the Cys\textsuperscript{147} and Cys\textsuperscript{148} residues was identified for both the oxidized and reduced forms of the enzyme (Fig. 2A). Analysis of the Asp\textsuperscript{131}–Arg\textsuperscript{143} fragment from the reduced form of human RNase H1 treated with NEM revealed a double NEM-labeled peptide, indicating that the Cys\textsuperscript{147} and Cys\textsuperscript{148} residues were accessible to the alkylating reagent (Fig. 4b). The Asp\textsuperscript{131}–Arg\textsuperscript{143} fragment of the oxidized form of human RNase H1 exhibited a mass consistent with the calculated mass for the peptide minus two hydrogen ions suggesting the formation of a disulfide bridge between the Cys\textsuperscript{147} and Cys\textsuperscript{148} residues (Fig. 4a). Consistent with this observation was the absence of the NEM-labeled Asp\textsuperscript{131}–Arg\textsuperscript{143} fragment for the oxidized/NEM form of human RNase H1, suggesting that no sulfhydryl moieties were present within the peptide (Fig. 4c). The lack of NEM label for this peptide also suggests that the disulfide bond between the adjacent cystine residues was present in the intact protein and therefore was not a tryptic peptide specific structure. Finally, larger peptides containing the Cys\textsuperscript{147} and Cys\textsuperscript{148} residues were also identified which were the products of one or more missed tryptic digestions. In all cases, the observed mass for these larger peptides was consistent with the formation of a disulfide bond between Cys\textsuperscript{147} and Cys\textsuperscript{148}, i.e. the calculated mass for the missed cleaved peptides minus two hydrogen ions.

The redox states of the remaining cysteine residues were also analyzed by μHPLC-ESI-FTICR mass spectrometry. Tryptic fragments Val\textsuperscript{11}–Arg\textsuperscript{19}, Thr\textsuperscript{37}–Arg\textsuperscript{47}, and Ala\textsuperscript{185}–Lys\textsuperscript{192} were identified that contained, respectively, Cys\textsuperscript{8}, Cys\textsuperscript{60}, and Cys\textsuperscript{191} (Fig. 2B). The oxidized form of the enzyme treated with NEM showed all three cysteine residues labeled with NEM (data not shown). In addition, a peptide mass corresponding the cross-linked fragments Val\textsuperscript{11}–Arg\textsuperscript{19} and Thr\textsuperscript{37}–Arg\textsuperscript{47} was also identified for the oxidized/NEM protein. The presence of both a cross-linked Val\textsuperscript{11}–Arg\textsuperscript{19}/Thr\textsuperscript{37}–Arg\textsuperscript{47} fragment and single NEM-labeled Val\textsuperscript{11}–Arg\textsuperscript{19} and Thr\textsuperscript{37}–Arg\textsuperscript{47} fragments suggests a partial or transient disulfide linkage between Cys\textsuperscript{18} and Cys\textsuperscript{46}. Clearly, this observed transient disulfide linkage was not contributing to redox dependent cleavage activity of human RNase H1 given the fact that the H1[D1–73] mutant, which did not contain the Cys\textsuperscript{18} and Cys\textsuperscript{46} residues, was also shown to be inactive under oxidized conditions.

The formation of a vicinal disulfide bridge between adjacent cysteines has been shown in peptides to result in a structure consisting of a novel eight-membered ring with either a cis- or trans-conformation (39, 40). This structure requires considerable distortion of the peptide backbone for its formation, and therefore, the occurrence of vicinal linkages are rare in proteins. One example is the quinoprotein methanol dehydrogenase from Methylobacterium extorquens (41). X-ray analysis of quinoprotein methanol dehydrogenase revealed a disulfide linkage between Cys\textsuperscript{266} and Cys\textsuperscript{282} in this case, the formation of the disulfide bridge produced an eight-membered ring with a cis-configuration and a non-planar linking peptide bond. Again, a significant distortion of the peptide backbone was observed with this structure. A perturbation of this nature in the human RNase H1 structure could account for the observed loss of cleavage activity for the enzyme under oxidized conditions.
Cys$^{147}$-Cys$^{148}$ Redox Switch of Human RNase H1

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