Effects of neutral salts and pH on the activity and stability of human RNase H2

Received January 26, 2017; accepted February 13, 2017; published online April 11, 2017

Misato Baba¹, Kenji Kojima¹, Rihoko Nakase¹, Shota Imai¹, Tomomi Yamasaki¹, Teisuke Takita¹, Robert J. Crouch² and Kiyoshi Yasukawa^{1,*}

¹Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Oiwakecho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan and ²Section on Formation of RNA, Division of Developmental Biology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA

*Kiyoshi Yasukawa, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Oiwakecho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan. Tel: +81-75-753-6268, Fax: +81-75-753-6265, email: yasukawa@kais.kyoto-u.ac.jp

Ribonuclease H (RNase H) specifically degrades the RNA of RNA/DNA hybrid. Recent study has shown that a single ribonucleotide is embedded in DNA double strand at every few thousand base pairs in human genome, and human RNase H2 is involved in its removal. Here, we examined the effects of neutral salts and pH on the activity and stability of human RNase H2. NaCl, KCl, RbCl and NaBr increased the activity to 170–390% at 10–60 mM, while LiCl, LiBr and CsCl inhibited it, suggesting that species of cation, but not anion, is responsible for the effect on activity. NaCl and KCl increased the stability by decreasing the first-order rate constant of the inactivation to 50–60% at 60–80 mM. The activity at 25–35 °C exhibited a narrow bell-shaped pH-dependence with the acidic and alkaline pK_e (pK_{e1} and pK_{e2}) values of 7.3–7.6 and 8.1–8.8, respectively. Enthalpy changes (ΔH°) of deprotonation were $5 \pm 21 \text{ kJ mol}^{-1}$ for pK_{e1} and $68 \pm 25 \text{ kJ mol}^{-1}$ for pK_{e2} . These results suggest that the ionizable groups responsible for pK_{e1} may be two out of Asp34, Glu35 and Asp141 of DEDD motif, and that for pK_{e2} may be Lys69 of DSK motif.

Keywords: activity; human RNase H2; neutral salt; pH dependence; stability.

Ribonucleases H (RNase H) is the enzyme that specifically hydrolyzes the 5'-phosphodiester bond of the RNA of RNA/DNA hybrids. RNase H is present ubiquitously ranging from bacteria to human. RNase H is classified into two groups, type I and type II. The former requires at least four consecutive ribonucleotides incorporated into DNA duplex for the degradation of RNA, while the latter requires only single ribonucleotide (1–3).

Various RNA/DNA hybrids are present in cells, and RNase H is involved in their processing. Such hybrids include template RNA/cDNA formed in retroviral replication, RNA primer/DNA formed in lagging strand synthesis and telomere RNA/DNA formed in telomere elongation. The hybrids identified in recent years are R-loop, a three-strand nucleic acid structure consisting of an RNA/DNA hybrid and a single DNA strand. Recent study has shown that single ribonucleotides are embedded in DNA double strand (4–6). Both type I and II RNases H have the activity to degrade the RNA strand of RNA/DNA hybrids, while only type II has the activity to incise single ribonucleotides, initiating the so-called ribonucleotide excision repair (RER) activity (1–3). The failure of the RNase H-mediated processing of RNA/DNA hybrids leads to double-strand DNA break (DSB) of chromosome (1–3). It was reported that DSB is repaired by homologous recombination (HR): cDNA is synthesized from the transcript RNA by reverse transcriptase, followed by the transcript RNA of the resulting RNA/DNA hybrid is degraded by RNase H and the resulting DNA fragment is used for HR with chromosomal DNA (7, 8).

Eukaryotic type II RNase H (RNase H2) is composed of three different subunits, one catalytic subunit (A) and two accessory subunits (B and C) (9). The active site has conserved DEDD and DSK motifs. The double mutation of Pro45→Asp and Tyr219→Ala in yeast RNase H2 abolishes the RER activity without impairing the RNA strand degrading activity (10). RNase H2 is not critical for viability in yeasts (11), while RNase H2 knock-out mice are embryonic lethal (12). In human, decrease in RNase H2 activity by the mutation of RNase H2 gene causes Aicardi–Goutières syndrome (AGS), a severe neuroinflammatory disorder (6, 13, 14). Recent study of the mechanism of AGS-RNase H2 onset has shown that nucleic acids are accumulated in cells, cGAS/STING works as a nucleic acid-sensing pathway and innate immune response is enhanced (15, 16).

Considering that RNase H2 is involved in various cellular events, the development of RNase H2 activators and inhibitors is an important subject. However, enzymatic characteristics of human RNase H2 remain to be elucidated. In this study, we examined the effects of neutral salts and pH on the activity and stability of recombinant human RNase H2 expressed in *Escherichia coli*.

Materials and Methods

Materials

The 3'-fluorescein-5-isothiocyanate (FITC)-labelled or 5'-4-((dimethylamino)-phenyl)-azo)-benzoic acid (Dabcyl)-labelled 18-mer nucleotides were purchased from Fasmac (Atsugi, Japan). All

other chemicals were of reagent grade and purchased from Nacalai Tesque (Kyoto, Japan).

Expression and purification of recombinant human RNase H2

Recombinant human RNase H2 was prepared as described previously (17). Briefly, *E. coli* BL21(DE3) [*F*⁻, *ompT*, *hsdS_B* (*r_B*-*m_B*-) *gal dem* (DE3)] was transformed with pET15b-hH2ABC, which was the pET-15b(+) plasmid (Merck Bioscience, Tokyo, Japan) harbouring the gene encoding A, B, and C subunits of human RNase H2 with a N-terminal (His)₆ tag at each subunit. The overnight culture of the transformants (15 ml) was added to 1500 ml of LB broth and in a 2-l flask and incubated at 30 °C under vigorous aeration by air-pump. When OD₆₆₀ reached 0.3, 1.2 ml of 0.5 M isopropyl β-D-1 thiogalactopyranoside (IPTG) was added and growth was continued at 30 °C for 3 h.

After centrifugation at 10,000 × *g* for 10 min, the cells were harvested, suspended with 20 ml of 20 mM Tris-HCl buffer (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol (DTT) (buffer A) and disrupted by sonication. After centrifugation at 20,000 × *g* for 40 min, the supernatant was collected and applied to a HiTrap Heparin HP column (GE Healthcare, Buckinghamshire, UK) previously equilibrated with buffer A. After the wash with buffer A containing 0.2 M NaCl, the bound RNase H2 was eluted with buffer A containing 0.4 M NaCl and applied to a HisTrap HP column (GE Healthcare), previously equilibrated with 20 mM Tris-HCl buffer (pH 7.5), 0.5 M NaCl (buffer B). After the wash with buffer B, the bound RNase H2 was eluted with buffer B containing 30 mM imidazole and applied to a PD-10 column (GE Healthcare) pre-equilibrated with buffer A. Purified enzyme solution was stored at -80 °C before use. The enzyme concentration was determined using the molar absorption coefficient at 280 nm of 83,030 M⁻¹ cm⁻¹.

GFC

Human RNase H2 (0.1 ml of 1.7 μM in 20 mM Tris-HCl buffer (pH 7.5)) was applied onto a COSMOSIL Packed Column 5Diol-300-II (7.5 mm inner diameter × 600 mm) (Nacalai Tesque) equilibrated with 20 mM Tris-HCl buffer (pH 7.5), 0.5 M L-Arg at a flow-rate of 1.0 ml/min and detected by absorbance at 280 nm (*A*₂₈₀).

Circular dichroism spectra measurement

Circular dichroism (CD) spectra of human RNase H2 (0.4 ml of 0.5 μM in 20 mM Tris-HCl buffer (pH 7.5)) was determined with a 2-mm cell using a J-820 spectropolarimeter (Jasco, Tokyo, Japan) under the following condition: spectral range 200–250 nm, 25 °C, 100 mdeg sensitivity, 0.2 nm resolutions, 4 s response time, 20 nm min⁻¹ scan rate and four accumulations. CD spectra were processed with a Jasco software, and finally expressed in mean-residue molar ellipticity units, [θ] (deg cm² dmol⁻¹).

RNase H2 assay

RNase H2 assay was performed using a fluorescence substrate as described previously (18, 19). Briefly, an RNA₁₈/DNA₁₈ hybrid (named R18/D18) was prepared by incubating 1.0 μl of 100 μM 3'-FITC-labelled 18-mer RNA 5'-gaucugagccugggagcu-FITC-3' (R18) and 1.2 μl of 100 μM 5'-DabcyI-labelled 18-mer DNA 5'-DabcyI-AG CTCCAGGCTCAGATC-3' (D18) in 50 mM Tris-HCl buffer (pH 8.0) containing 60 mM KCl at 25 °C for 30 min. A hybrid consisting of DNA₁₄-RNA₁-DNA₃ and DNA₁₈ (named R1/D18) was prepared as described above by using 3'-FITC-labelled 5'-GATCTGA GCCTGGGAGCT-FITC-3' (R1) instead of R18.

Enzyme reaction was started by adding 20 μl of 0.33 or 3 nM RNase H2 to the 180 μl of the mixture containing 2.8 or 5.6 nM substrate (R18/D18 or R1/D18) in a 96-well plate. The reaction buffers were 50 mM Tris-HCl buffer at pH 7.0–8.8 or 50 mM 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropane sulfonic acid (AMPSO)-NaOH buffer at pH 8.6–10.4, each containing 5 mM MgCl₂. The reaction was carried out at 25 °C, 30 °C or 35 °C, each with the initial enzyme and R1/D18 concentrations of 33 or 300 pM and 2.5 or 5.0 nM, respectively. The reaction was monitored by following the increase in fluorescence intensity at 515 nm with excitation at 490 nm with an EnSight (PerkinElmer, Waltham, MA) every 20 s for 10 min.

The Michaelis–Menten equation was expressed as $v_o = (k_{cat}/K_m)[E]_o[S]_o$, where v_o , k_{cat} , $[E]_o$ and $[S]_o$ are the initial reaction rate, the molecular activity, the initial enzyme concentration and

the initial substrate concentration, respectively. The proton dissociation constants (K_{e1} and K_{e2}) for the bell-shaped pH-dependence of the activity (k_{cat}/K_m) were calculated from Equation (1) or (2) by a non-linear least squares regression method with Kaleida Graph Version 3.5 (Synergy Software, Essex, VT):

$$(k_{cat}/K_m)_{obs} = (k_{cat}/K_m)_o / \{1 + ([H]/K_{e1}) + (K_{e2}/[H])\} \quad (1)$$

$$(k_{cat}/K_m)_{obs} = (k_{cat}/K_m)_o / \{1 + ([H]^2/K_{e1}^2) + ([H]/K_{e1}) + (K_{e2}/[H])\} \quad (2)$$

In this equation, $(k_{cat}/K_m)_o$, $(k_{cat}/K_m)_{obs}$ and $[H]$ are the intrinsic k_{cat}/K_m value, the k_{cat}/K_m value observed and the proton concentration, respectively, at a specified pH.

Irreversible thermal inactivation of human RNase H2

Human RNase H2 (330 pM) was incubated at 30 °C, 35 °C or 40 °C in 20 mM Tris-HCl buffer containing various salts for specified durations followed by the incubation on ice for 5 min. The remaining activity was determined at 25 °C as described above.

Assuming that the thermal inactivation reaction of human RNase H2 is irreversible and consists of only one step, the first-order rate constant, k_{obs} , of the thermal inactivation was evaluated by plotting logarithmic values of the residual activity against the time of heat treatment according to Equation (3), as described previously (20, 21)

$$\ln B = A - k_{obs}t \quad (3)$$

where A is the constant term and B is the relative activity (%) defined as the ratio of the initial reaction rate at a time for the thermal incubation ($= t$) to that without the incubation.

Thermodynamic analysis

The enthalpy change, ΔH° , of deprotonation was determined from a van't Hoff plot according to Equation (4), as described previously (22). The activation energy, E_a , for the thermal inactivation was determined from an Arrhenius plot according to Equation (5), as described previously (20, 21)

$$\ln(K_e) = A - (\Delta H^\circ/R)(1/T) \quad (4)$$

$$\ln(k_{obs}) = A - (E_a/R)(1/T) \quad (5)$$

where A , R and T are the constant term, the gas constant ($= 8.314 \text{ J K}^{-1} \text{ mol}^{-1}$) and absolute temperature in degrees Kelvin, respectively.

The Gibbs free energy change of activation, $\Delta G^\ddagger$, and the enthalpy change of activation, $\Delta H^\ddagger$, the entropy change of activation, $\Delta S^\ddagger$, for the thermal inactivation at certain temperature were determined according to Equations (6), (7) and (8), respectively:

$$\Delta G^\ddagger = -RT[\ln(k_{obs}) - \ln(RT/Nh)] \quad (6)$$

$$\Delta H^\ddagger = E_a - RT \quad (7)$$

$$\Delta S^\ddagger = (\Delta H^\ddagger - \Delta G^\ddagger)/T \quad (8)$$

where A , R , T , N and h are the constant term, the gas constant ($= 8.314 \text{ J K}^{-1} \text{ mol}^{-1}$), absolute temperature in Kelvin, Avogadro number ($= 6.022 \times 10^{23} \text{ mol}^{-1}$) and Plank constant ($= 6.626 \times 10^{-34} \text{ J s}$), respectively.

Results and Discussion

Production of recombinant human RNase H2

Human RNase H2 is composed of three different subunits (A, B and C). The human RNase H2 expression plasmid, pET15b-hH2ABC, has a T7 promoter sequence followed by genes for A, B and C, each containing (His)₆ tag at its N-terminus (17). These three subunits were co-expressed in the BL21(DE3) cells transformed with pET15b-hH2ABC. The active enzyme was purified from the soluble fractions of the

cells with heparin affinity chromatography as the first step and Ni^{2+} affinity column chromatography as the second step. It is noted that human RNase H2 was eluted at relatively low imidazole concentration (30 mM). Fig. 1 shows the results of SDS-PAGE analysis of the active fractions at each purification stage and the purified enzyme preparations. The purified preparation yielded three bands corresponding to A, B and C. Figs S1 and S2 (see online supplementary material for a colour version of this figure) show the elution pattern of gel filtration chromatography (GFC) and CD spectrum, respectively, of the purified enzyme preparation. In GFC, L-Arg (0.5 M) was contained in the equilibrium buffer to improve resolution (23). Human RNase H2 was eluted at the position corresponding to molecular mass of 120 kDa (Fig. S1, see online supplementary material for a colour version of this figure). On SDS-PAGE, the peak fractions showed three bands with the same intensity, indicating that the purified enzyme preparation consists of each one molecule of A, B and C (Fig. S1D, see online supplementary material for a colour version of this figure). The molecular mass of the heterotrimeric complex is calculated to be 88 kDa based on the amino acid sequence, and the difference from the value estimated from GFC analysis (120 kDa) might be derived from the unique character of human RNase H2. On CD spectroscopy, the purified enzyme preparation exhibited negative ellipticities at around 200–250 nm with minimum values around 208 nm (Fig. S2, see online supplementary material for a colour version of this figure).

Effects of salts on the human RNase H2 activity

To analyze the effects of various neutral salts on the human RNase H2 activity, we performed fluorescence-based RNase H2 assay. An RNA₁₈/DNA₁₈ hybrid (R18/D18) (Fig. 2A) and a hybrid consisting of DNA₁₄–RNA₁–DNA₃ and DNA₁₈ (R1/D18)

Fig. 1 SDS-PAGE under reducing conditions. Coomassie brilliant blue-stained 12.5% SDS-polyacrylamide gel showing marker proteins (Protein Molecular Weight Marker (Broad), Takarabio, Otsu, Japan) (lane M), soluble fractions of the total extracts (lane 1), active fractions of heparin affinity chromatography (lane 2), and active fractions of Ni^{2+} affinity chromatography, which was purified enzyme preparations of human RNase H2 (lane 3). A, B and C subunits of human RNase H2 are indicated by arrows.

(Fig. 3A) were used as the substrate. R18/D18 is designed to emit fluorescence when RNA₁₈ is cleaved at a site close to the 3' end, and the fluorescein-labelled RNA fragment dissociates from the complementary DNA strand. R1/D18 is designed to emit fluorescence when DNA₁₄–RNA₁–DNA₃ is cleaved at the 5'-end of the RNA₁.

In the reaction with R18/D18, the relative activity of human RNase H2, which is defined as the ratio of the activity in the presence of salt to that in the absence, increased with increasing concentrations of NaCl, KCl, RbCl or NaBr, reached the highest (180–390%) at 30–60 mM and decreased (Fig. 2B). As is the case with R1/D18, the relative activity increased with increasing concentrations of NaCl, KCl, RbCl or NaBr, reached the highest (170–250%) at 10–30 mM and decreased (Fig. 3B). It is noted that the result with NaCl is in good agreement with those reported previously (10). In contrast, the relative activity decreased with increasing concentrations of LiCl, LiBr or CsCl. The IC_{50} values of LiCl, LiBr and CsCl in the reaction with R18/D18 were 32, 43 and 26 mM, respectively

A

B

C

Fig. 2 Effects of salts on the human RNase H2 activity to cleave the RNA₁₈ strand. (A) Sequences of the RNA–DNA hybrid substrate R18/D18. (B, C) Activity versus salt concentration. The reaction was carried out in 50 mM Tris–HCl buffer (pH 8.0), 5 mM MgCl_2 at 37°C. The initial enzyme and substrate concentrations were 33 pM and 2.5 nM, respectively. The relative activity is the ratio of the activity in the presence of salt to that in the absence of salt. Average values of triplicate experiments with SD value are shown.

Fig. 3 Effects of salts on the human RNase H2 activity to cleave the DNA₁₄–RNA₁–DNA₃–strand. (A) Sequences of the RNA–DNA hybrid substrate R1/D18. (B, C) Activity versus salt concentration. The reaction was carried out and analyzed as described in Fig. 2.

(Fig. 2C), and those in the reaction with R1/D18 were 51, 48 and 40 mM, respectively (Fig. 3C). These results suggest that species of cation, rather than anion, might be responsible for the effect on activity.

Effects of salts on the human RNase H2 stability

To analyze the effect of NaCl on the human RNase H2 stability, we incubated human RNase H2 at 30°C, 35°C or 40°C for specified durations and measured the remaining activity by the assay using R18/D18. The natural logarithm of the relative activity (%) plotted against the incubation time gave linear relationships at all temperatures (Fig. 4A–C), indicating that the inactivation followed first-order kinetics. The first-order rate constant (k_{obs}) values at 30°C, 35°C and 40°C in the presence of 10 mM NaCl were 7.1×10^{-4} , 1.7×10^{-3} and $4.7 \times 10^{-3} \text{ s}^{-1}$, respectively, and those in the absence were 7.7×10^{-4} , 2.7×10^{-3} and $7.0 \times 10^{-3} \text{ s}^{-1}$, respectively, indicating that 10 mM NaCl stabilized human RNase H2. Fig. 4D shows an Arrhenius plot. The natural logarithm of k_{obs} against $1/T$ showed a linear relationship, and the activation energies (E_a) of thermal inactivation were calculated from the slope to be $149 \pm 5 \text{ kJ mol}^{-1}$ for the presence of 10 mM NaCl and $175 \pm 13 \text{ kJ mol}^{-1}$ for the absence.

To analyze the effects of NaCl and KCl concentrations on the human RNase H2 stability, we incubated human RNase H2 at 37°C for 10 min in the presence of 0–200 mM NaCl or KCl, and measured the remaining activity by the assay using R18/D18. The k_{obs} values decreased with increasing NaCl or KCl concentrations and reached 40% at 80 mM NaCl or 52% at 60 mM KCl (Fig. 5A). The half-life ($t_{1/2}$) at 37°C was 3.8 min in the absence of salt. It increased with increasing NaCl or KCl concentrations and reached 9.5 min at 80 mM NaCl or 7.3 min at 60 mM KCl (Fig. 5B).

To analyze the inactivation mechanism, we incubated human RNase H2 with or without 10 mM NaCl at 40°C for 3 min and measured their elution patterns in GFC and their CD spectra. There is little difference in the elution patterns of human RNase H2s before and after the incubation at 40°C (Fig. S1, see online supplementary material for a colour version of this figure). On the other hand, the $[\theta]_{208}$ and $[\theta]_{222}$ values of human RNase H2 were in the order of before the incubation < after the incubation with 10 mM NaCl < after incubation without NaCl (Fig. S2, see online supplementary material for a colour version of this figure). These results suggest that the inactivation results from the denaturation rather than the dissociation of the heterotrimeric complex and that NaCl stabilizes human RNase H2 by suppressing the denaturation.

Mechanism of activation of human RNase H2 by salts

The feature of the activation of human RNase H2 by NaCl, KCl, RbCl and NaBr is that the 1.7–3.9-fold activation was observed at very low-salt concentration (10–60 mM) (Figs 2B and 3B). Similar to *Thermotoga maritima* type 2 RNase H (RNase HII), the 3-fold activation was observed at 50 mM NaCl (9). In the following, we discuss the differences between the activation of human RNase H2 and those of a typical halophilic enzyme, *Halobacterium* sp. NRC-1 RNase H1 (Halo-RNase H1) (24) and *Bacillus thermoproteolyticus* zinc metalloproteinase thermolysin (25). The Halo-RNase H1 activity requires the presence of 2 M NaCl, which was thought to result from the salt-mediated unfavourable suppression of negative charge repulsion at the molecular surface including the active site (24). The thermolysin activity increased in an exponential fashion with increasing concentration of NaCl and reached 1300–1500% at 4 M, which was thought to result from the interaction between respective ions and particular residues and/or the salt-mediated suppression of unfavourable charge repulsion (25). It is noted that there is almost no activation at 50 mM NaCl in Halo-RNase H1 or thermolysin, much different from the case with human RNase H2.

Shaban *et al.* (26) reported the crystal structure of mouse RNase H2. They created a model for the complex of mouse RNase H2 and the RNA/DNA hybrid using the crystal structure of the complex of *Bacillus halodurans* RNase H1 and the RNA/DNA hybrid as a starting model and showed that unlike the case with *B. halodurans* RNase H1, the RNA/DNA hybrid makes significant clash with mouse RNase H2 subunit A (26).

Fig. 4 Irreversible thermal inactivation of human RNase H2. Human RNase H2 (330 pM) was incubated at 30 (A), 35 (B) or 40 °C (C) in 20 mM Tris-HCl buffer (pH 7.5) in the presence or absence of 10 mM NaCl for the indicated durations. Then, the reaction was carried out using R18/D18 as described in Fig. 2. The relative activity is the ratio of the initial reaction rate with incubation for the indicated durations to that without incubation. (D) Arrhenius plots of k_{obs} values. The logarithm of k_{obs} values were plotted against the reciprocal of the absolute temperature. Activation energies (E_a) of thermal inactivation were calculated from the slope to be $149 \pm 5 \text{ kJ mol}^{-1}$ for the presence of 10 mM NaCl and $175 \pm 13 \text{ kJ mol}^{-1}$ for the absence.

In the current study, the optimum concentrations of NaCl, KCl, RbCl and NaBr were different according to the substrate (60, 60, 30 and 40 mM, respectively, for R18/D18 (Fig. 2B) and 20, 30, 20 and 20 mM, respectively, for R1/D18 (Fig. 3B)). These evidences suggest that the salt-based activation of human RNase H2 results from the interaction between respective ions and particular residues as well as that between respective ions and the RNA/DNA hybrid.

Mechanism of inhibition of human RNase H2 by salts

NaCl, KCl, RbCl and NaBr inhibited the human RNase H2 activity at 50–200 mM (Figs 2B and 3B), and LiCl, LiBr and CsCl inhibited it at 0–200 mM (Figs 2C and 3C). The inhibition by NaCl and LiCl was reported in TREX1 (27). TREX1 is a 3'→5' exonuclease in mammalian cells. Like RNases H, TREX1 has a DEDD motif and two divalent metal ions catalytic system. In addition, decrease in TREX1 activity by the mutation of its gene causes AGS (6). Brucet *et al.* proposed that based on the crystal structure of the complex of TREX1 and a nucleotide, LiCl and NaCl inhibited the TREX1 activity by competing with the divalent cations for the binding at the active site (27). Similar inhibition of enzyme activity by LiCl was reported in inositol monophosphate (28). If such mechanism can be applied to human RNase H2, our results suggest that Na⁺, K⁺ and Rb⁺ exhibit weak inhibition because they have low binding ability with the active site, while Li⁺ and Cs⁺ exhibit strong inhibition because they have high binding ability with the active site.

Mechanism of stabilization of human RNase H2 by salts

Based on the results of thermal inactivation (Fig. 4) and according to Equations (5)–(8), E_a , the Gibbs free energy change of activation ($\Delta G^\ddagger$), the enthalpy change of activation ($\Delta H^\ddagger$) and the entropy change of activation ($\Delta S^\ddagger$) for thermal inactivation were 175 kJ mol^{-1} , 92 kJ mol^{-1} , 172 kJ mol^{-1} and $265 \text{ J mol}^{-1} \text{ K}^{-1}$, respectively, in the absence of NaCl and were 149 kJ mol^{-1} , 93 kJ mol^{-1} , 146 kJ mol^{-1} and $178 \text{ J mol}^{-1} \text{ K}^{-1}$, respectively, in the presence of 10 mM NaCl. This indicates that the presence of 10 mM NaCl decreased $\Delta H^\ddagger$ and $\Delta S^\ddagger$, suggesting that the enhanced thermal stability by salt was due to the decrease in $\Delta S^\ddagger$.

The highest stabilization of human RNase H2 was observed at 80 mM NaCl or 60 mM KCl (Fig. 5), which is similar concentration to the case with the activation (Figs 2B and 3B). This suggests that the mechanism of stabilization by salts is similar to that of activation. On the other hand, human RNase H2 almost lacked activity at 200 mM NaCl or KCl (Figs 2B and 3B), while it exhibited activity after incubation at 37 °C for 10 min at 200 mM NaCl or KCl (Fig. 5B). This suggests that the binding of NaCl and KCl to the active site inhibits activity, but is reversible.

Effects of pH on the RNase H2 activity

To analyze the effects of pH on the RNase H2 activity, we measured the activity at pH 7.0–10.4 at 25 °C, 30 °C and 35 °C by the assay using R1/D18. The k_{cat}/K_m values plotted against pH showed a narrow

Fig. 5 Effects of salts on the human RNase H2 thermostability. Human RNase H2 (330 pM) was incubated at 37 °C in the presence of 0–200 mM NaCl or KCl for specified durations. Then, the human RNase H2 reaction was carried out using R18/D18 as described in Fig. 2. (A) Relative k_{obs} versus salt concentration. The relative k_{obs} is the ratio of the k_{obs} for the incubation of indicated salt concentration to that for the incubation without salts. (B) $t_{1/2}$ versus salt concentration. The $t_{1/2}$ is the time required to reduce initial activity by 50% by the incubation at 37 °C. Error bars indicate SD values of triplicate measurements.

bell-shaped profiles, which is in good agreement with those reported previously (10). First we applied the results to the reaction scheme with two ionizable groups (Fig. S3A, see online supplementary material for a colour version of this figure), which is generally used for the analysis of pH-dependence of enzyme activity. The $(k_{cat}/K_m)_o$ value and the pK_{e1} and pK_{e2} values were calculated according to Equation (1). However, the curve expressed by Equation (1) did not fit the data points (Fig. S3B, see online supplementary material for a colour version of this figure), indicating that this conventional reaction scheme with two ionizable groups (each at acidic and alkaline side) involved in enzyme activity was not applicable to pH dependence of human RNase H2 activity. Assuming that the steep increase in $(k_{cat}/K_m)_{obs}$ at pH 7.0–8.0 results from the presence of two ionizable groups with the same pK_a values, we applied the results to the reaction scheme with three ionizable groups, in which only the EHS complex gives products (Fig. 6A). The $(k_{cat}/K_m)_o$ value and the pK_{e1} and pK_{e2} values were calculated according to Equation (2). The curve expressed by Equation (2) fit the data points well (Fig. 6B), indicating that this scheme was applicable. Fig. 6C shows a van't Hoff plot. Enthalpy changes

(ΔH°) of deprotonation were $5 \pm 21 \text{ kJ mol}^{-1}$ for pK_{e1} and $68 \pm 25 \text{ kJ mol}^{-1}$ for pK_{e2} .

Catalytic mechanism of human RNase H2

DEDD and DSK motifs are conserved in RNase H2. Shaban *et al.* (26) showed that based on the crystal structure of mouse RNase H2, Asp34, Glu35, Asp142 and Asp170 likely coordinates two divalent metal ions. Rychlik *et al.* (9) showed that based on the crystal structure of the complex of a single peptide *T. maritima* RNase HII with a nucleic acid, Asp18, Glu19 and Asp107 of DEDD motif coordinate metal ions, while Asp124 of DEDD motif does not. Consistent with that, the mutation of counterparts of Asp18, Glu19 or Asp107 abolished the activities of RNases HII from *Archaeoglobus fulgidus* and *Thermococcus kodakaraensis*, while the mutation of Asp124 did not (29, 30). Rychlik *et al.* (9) also showed that Lys47 of DSK motif interacts with the scissile phosphate. Based on the crystal and modelled structural analysis of the complex of human RNase H2 with a nucleic acid and the mutational analysis, Figiel *et al.* (31) showed the followings: (i) like *T. maritima* RNase HII, Asp34, Glu35 and Asp141 of DEDD motif coordinates metal ions, while Asp169 of DEDD motif does not; (ii) the DSK motif-containing loop is located close to the active site and is flexible in the absence of substrate; and (iii) the mutation of Lys69 of the DSK motif abolished activity. In Fig. 6A, "EH" is the free enzyme that can bind the substrate and give the product. These results suggest that the "EH" corresponds to the human RNase H2 in which Asp34, Glu35 and Asp141 are deprotonated and Lys69 are protonated. They also suggested that at least one residue out of Asp34, Glu35 and Asp141 has an ionizable group involved in the catalytic mechanism responsible for acidic pK_e (pK_{e1}) and Lys69 may be that for alkaline pK_e (pK_{e2}). pK_e values are commonly used for the estimation of ionizable groups involved in the catalytic mechanism. In this study, we used not only pK_e but also ΔH° of deprotonation for the estimation for the following two reasons: (i) pK_a of active-site residues sometimes vary considerably depending on the microenvironment. For example, the pK_a values of the active-site glutamate were 8.4 in xylanase (32) and 7.0 in carboxypeptidase A (33); (ii) each amino acid has its own ΔH° values of side chain, and such ΔH° values of amino acid residues in protein are little affected by the microenvironment and are almost equal to those of free amino acids (22, 34). Activity at 25–35 °C exhibited a narrow bell-shaped pH-dependence with the acidic and alkaline pK_e (pK_{e1} and pK_{e2}) values of 7.3–7.6 and 8.1–8.8, respectively (Fig. 6A), which is similar to those of RNases HII from *E. coli* (35) and *T. maritima* (30).

In this study, the conventional reaction scheme with two ionizable groups (each at acidic and alkaline side) involved in enzyme activity was not applicable to pH dependence of human RNase H2 activity (Fig. S3, see online supplementary material for a colour version of this figure). On the other hand, the one with three ionizable groups (two at acidic side and one at alkaline side) was applicable (Fig. 6). In this scheme, pK_{e1} and

Fig. 6 Effect of pH on the human RNase H2 activity. (A) Reaction scheme with three ionizable groups responsible for activity. Only the EHS complex gives products. (B) $(k_{cat}/K_m)_{obs}$ versus pH. The reaction was carried out at 25°C (open circle), 30°C (open square) and 35°C (open triangle) with the initial enzyme and R1/D18 concentrations of 300 pM and 5.0 nM, respectively. Solid line represents a curve expressed by Equation (2) with the $(k_{cat}/K_m)_o$, pK_{e1} and pK_{e2} values of $(1.8 \pm 0.2) \times 10^7 \text{ M}^{-1} \text{s}^{-1}$, 7.7 ± 0.1 and 8.9 ± 0.1 , respectively, for 25°C, $(2.7 \pm 0.2) \times 10^7 \text{ M}^{-1} \text{s}^{-1}$, 7.4 ± 0.1 , and 8.8 ± 0.1 , respectively, for 30°C, and $(3.4 \pm 0.4) \times 10^7 \text{ M}^{-1} \text{s}^{-1}$, 7.5 ± 0.1 , and 8.3 ± 0.1 , respectively, for 35°C, which was drawn to fit the experimental data. (C) van't Hoff plot of pK_e values. pK_{e1} and pK_{e2} values were plotted against the reciprocal of the absolute temperature. Enthalpy changes (ΔH°) of deprotonation were calculated from the slope to be $5 \pm 21 \text{ kJ mol}^{-1}$ for pK_{e1} and $68 \pm 25 \text{ kJ mol}^{-1}$ for pK_{e2} .

pK_{e2} values were calculated 7.3–7.6 and 8.1–8.8, respectively, and ΔH° of deprotonation was calculated $5 \pm 21 \text{ kJ mol}^{-1}$ for pK_{e1} and $68 \pm 25 \text{ kJ mol}^{-1}$ for pK_{e2} (Fig. 6). It should be noted that the ΔH° of deprotonation for pK_{e1} ($5 \pm 21 \text{ kJ mol}^{-1}$) is similar to that of the side chains of L-Asp (-6 to 6 kJ mol^{-1}) (34), suggesting that the ionizable groups responsible for pK_{e1} may be two residues out of Asp34, Glu35 and Asp141 of DEDD motif. We speculate that the difference between the pK_{e1} of human RNase H2 (7.3–7.6) and the pK_a of the side chain of Asp in protein (around 3–5) might be due to the microenvironment in the active site, as discussed above. On the other hand, the pK_{e2} value of human RNase H2 (8.1–8.8) is similar to the pK_a of Lys in protein (around 8–10), and the ΔH° of deprotonation for pK_{e2} of human RNase H2 ($68 \pm 25 \text{ kJ mol}^{-1}$) was similar to the ΔH° of the side chain of Lys ($70.4 \pm 4.4 \text{ kJ mol}^{-1}$) (34). This suggests that the ionizable group responsible for pK_{e2} may be Lys69.

Conclusions

NaCl, KCl, RbCl and NaBr increased the human RNase H2 activity, while LiCl, LiBr and CsCl inhibited it. The ionizable groups responsible for pK_{e1} may be two out of Asp34, Glu35 and Asp141 of DEDD motif, and that for pK_{e2} may be Lys69 of

DSK motif. Further study is required to explore the mechanisms of the activation and stabilization of human RNase H2 by salts and to assign its ionizable residues controlling the activity. On the other hand, the results presented in this study might be useful to establish the reaction condition in the screening of activators and inhibitors of human RNase H2.

Supplementary Data

Supplementary Data are available at *JB* Online.

Funding

This study was supported in part by Grants-in-Aid for Scientific Research (No. 21580110) from the Japan Society for the Promotion of Science (K.K. and K.Y.), the Salt Science Research Foundation (K.K. and K.Y.) and the IRP of NICHHD (R.J.C.).

Conflict of Interest

None declared.

References

1. Cerritelli, S. M., and Crouch, R. J. (2009) Ribonuclease H: the enzymes in eukaryotes. *FEBS J.* **276**, 1494–1505
2. Champoux, J. J., and Schultz, S. J. (2009) Ribonuclease H: properties, substrate specificity and roles in retroviral reverse transcription. *FEBS J.* **276**, 1506–1516

3. Tadokoro, T., and Kanaya, S. (2009) Ribonuclease H: molecular diversities, substrate binding domains, and catalytic mechanism of the prokaryotic enzymes. *FEBS J.* **276**, 1482–1493
4. Sparks, J. L., Chon, H., Cerritelli, S. M., Kunkel, T. A., Johansson, E., Crouch, R. J., and Burgers, P. M. (2012) RNase H2-initiated ribonucleotide excision repair. *Mol. Cell* **47**, 980–986
5. Williams, J. S., and Kunkel, T. A. (2014) Ribonucleotides in DNA: origins, repair and consequences. *DNA Repair* **19**, 27–37
6. Cerritelli, S. M., and Crouch, R. J. (2016) The balancing act of ribonucleotides in DNA. *Trends Biochem. Sci.* **41**, 434–445
7. Storici, F., Bebenek, K., Kunkel, T. A., Gordenin, D. A., and Resnick, M. A. (2007) RNA-templated DNA repair. *Nature* **447**, 338–341
8. Keskin, H., Shen, Y., Huang, F., Patel, M., Yang, T., Ashley, K., Mazin, A. V., and Storici, F. (2014) Transcript-RNA-templated DNA recombination and repair. *Nature* **515**, 436–439
9. Rychlik, M. P., Chon, H., Cerritelli, S. M., Klimek, P., Crouch, R. J., and Nowotny, M. (2010) Crystal structures of RNase H2 in complex with nucleic acid reveal the mechanism of RNA-DNA junction recognition and cleavage. *Mol. Cell* **40**, 658–670
10. Chon, H., Sparks, J. L., Rychlik, M., Nowotny, M., Burgers, P. M., Crouch, R. J., and Cerritelli, S. M. (2013) RNase H2 roles in genome integrity revealed by unlinking its activities. *Nucleic Acids Res.* **41**, 3130–3143
11. Arudchandran, A., Cerritelli, S., Narimatsu, S., Itaya, M., Shin, D. Y., Shimada, Y., and Crouch, R. J. (2000) The absence of ribonuclease H1 or H2 alters the sensitivity of *Saccharomyces cerevisiae* to hydroxyurea, caffeine and ethyl methanesulphonate: implications for roles of RNase H in DNA replication and repair. *Genes Cells* **5**, 789–802
12. Reijns, M. A., Rabe, B., Rigby, R. E., Mill, P., Astell, K. R., Lettice, L. A., Boyle, S., Leitch, A., Keighren, M., Kilanowski, F., Devenney, P. S., Sexton, D., Grimes, G., Holt, I. J., Hill, R. E., Taylor, M. S., Lawson, K. A., Dorin, J. R., and Jackson, A. P. (2012) Enzymatic removal of ribonucleotides from DNA is essential for mammalian genome integrity and development. *Cell* **149**, 1008–1022
13. Crow, Y. J., Leitch, A., Hayward, B. E., Garner, A., Parmer, R., Griffith, E., Ali, M., Semple, C., Aicardi, J., Babul-Hirji, R., Baumann, C., Baxter, P., Bertini, E., Chandler, K. E., Chitayat, D., Cau, D., Déry, C., Fazzi, E., Goizet, C., King, M. D., Klepper, J., Lacombe, D., Lanzi, G., Lyall, H., Martínez-Frías, M. L., Mathieu, M., McKeown, C., Monier, A., Oade, Y., Quarrel, O. W., Rittey, C. D., Rogers, R. C., Sanchis, A., Stephenson, J. B., Tacke, U., Till, M., Tolmie, J. L., Tomlin, P., Voit, T., Weschke, B., Woods, C. G., Lebon, P., Bonthron, D. T., Ponting, C. P., and Jackson, A. P. (2006) Mutations in genes encoding ribonuclease H2 subunits cause Aicardi–Goutières syndrome and mimic congenital viral brain infection. *Nat. Genet.* **38**, 910–916
14. Coffin, S. R., Hollis, T., and Perrion, F. W. (2011) Functional consequences of the RNase H2A subunit mutations that cause Aicardi–Goutières syndrome. *J. Biol. Chem.* **286**, 16984–16991
15. Mackenzie, K. J., Carroll, P., Lettice, L., Tarnauskaite, Ž., Reddy, K., Dix, F., Revuelta, A., Abbondati, E., Rigby, R. E., Rabe, B., Kilanowski, F., Grimes, G., Fluteau, A., Devenney, P. S., Hill, R. E., Reijns, M. A., and Jackson, A. P. (2016) Ribonuclease H2 mutations induce a cGAS/STING-dependent innate immune response. *EMBO J.* **35**, 831–844
16. Pokatayev, V., Hasin, N., Chon, H., Cerritelli, S. M., Sakhuja, K., Ward, J. M., Morris, H. D., Yan, N., and Crouch, R. J. (2016) RNase H2 catalytic core Aicardi–Goutières syndrome-related mutant invokes cGAS–STING innate immune-sensing pathway in mice. *J. Exp. Med.* **7**, 329–336
17. Chon, H., Vassilev, A., DePamphilis, M. L., Zhao, Y., Zhang, J., Burgers, P. M., Crouch, R. J., and Cerritelli, S. M. (2009) Contributions of the two accessory subunits, RNASEH2B and RNASEH2C, to the activity and properties of the human RNase H2 complex. *Nucleic Acids Res.* **37**, 96–110
18. Parniak, M. A., Min, K. L., Budihis, S. R., Le Grice, S. F. J., and Beutler, J. A. (2003) A fluorescence-based high-throughput screening assay for inhibitors of human immunodeficiency virus-1 reverse transcriptase-associated ribonuclease H activity. *Anal. Biochem.* **322**, 33–39
19. Nishimura, K., Yokokawa, K., Hisayoshi, T., Fukatsu, K., Kuze, I., Konishi, A., Mikami, B., Kojima, K., and Yasukawa, K. (2015) Preparation and characterization of the RNase H domain of Moloney murine leukemia virus reverse transcriptase. *Protein Expr. Purif.* **113**, 44–50
20. Yasukawa, K., Nemoto, D., and Inouye, K. (2008) Comparison of the thermal stabilities of reverse transcriptases from avian myeloblastosis virus and Moloney murine leukemia virus. *J. Biochem.* **143**, 261–268
21. Yasukawa, K., Mizuno, M., Konishi, A., and Inouye, K. (2010) Increase in thermal stability of Moloney murine leukaemia virus reverse transcriptase by site-directed mutagenesis. *J. Biotechnol.* **150**, 299–306
22. Morishima, A., Yasukawa, K., and Inouye, K. (2012) A possibility of a protein-bound water molecule as the ionizable group responsible for pK_e at the alkaline side in human matrix metalloproteinase 7 (MMP-7) activity. *J. Biochem.* **151**, 501–509
23. Arakawa, T., Ejima, D., Tsumoto, K., Ishibashi, M., and Tokunaga, M. (2007) Improved performance of column chromatography by arginine: dye-affinity chromatography. *Protein Exp. Purif.* **52**, 410–414
24. You, D.-J., Jongruja, N., Tannous, E., Angkawidjaja, C., Koga, Y., and Kanaya, S. (2014) Structural basis for salt-dependent folding of ribonuclease H1 from halophilic archaeon *Halobacterium* sp. NRC-1. *J. Struct. Biol.* **187**, 119–128
25. Inouye, K., Lee, S.-B., Nambu, K., and Tonomura, B. (1997) Effects of pH, temperature, and alcohols on the remarkable activation of thermolysin by salts. *J. Biochem.* **122**, 358–364
26. Shaban, N. M., Harvey, S., Perrino, F. W., and Hollis, T. (2010) The structure of the mammalian RNase H2 complex provides insight into RNA–DNA hybrid processing to prevent immune dysfunction. *J. Biol. Chem.* **285**, 3617–3624
27. Brucet, M., Querol-Audi, J., Bertlik, K., Lloberas, J., Fita, I., and Celada, A. (2008) Structural and biochemical studies of TREX1 inhibition by metals. Identification of a new active histidine conserved in DEDDh exonucleases. *Protein Sci.* **17**, 2059–2069
28. Pollack, S. J., Atack, J. R., Knowles, M. R., McAllister, G., Ragan, C. I., Baker, R., Fletcher, S. R., Iversen, L. L., and Broughton, H. B. (1994) Mechanism of inositol monophosphatase, the putative target of lithium therapy. *Proc. Natl. Acad. Sci. USA* **91**, 5766–5770

29. Chapados, B. R., Chai, Q., Hosfield, D. J., Qiu, J., Shen, B., and Tainer, J. A. (2001) Structural biochemistry of a type 2 RNase H: RNA primer recognition and removal during DNA replication. *J. Mol. Biol.* **307**, 541–556
30. Muroya, A., Tsuchiya, D., Ishikawa, M., Haruki, M., Morikawa, M., Kanaya, S., and Morikawa, K. (2001) Catalytic center of an archaeal type 2 ribonuclease H as revealed by X-ray crystallographic and mutational analyses. *Protein Sci.* **10**, 707–714
31. Figiel, M., Chon, H., Cerritelli, S. M., Cybulska, M., Crouch, R. J., and Nowotny, M. (2011) The structural and biochemical characterization of human RNase H2 complex reveals the molecular basis for substrate recognition and Aicardi–Goutières syndrome defects. *J. Biol. Chem.* **286**, 10540–10550
32. Joshi, M. D., Sidhu, G., Pot, I., Brayer, G. D., Withers, S. G., and McIntosh, L. P. (2000) Hydrogen bonding and catalysis: a novel explanation for how a single amino acid substitution can change the pH optimum of a glycosidase. *J. Mol. Biol.* **299**, 255–279
33. Zhang, K., and Auld, D. S. (1995) Structure of binary and ternary complexes of zinc and cobalt carboxypeptidase A as determined by X-ray absorption fine structure. *Biochemistry* **34**, 16306–16312
34. Takeharu, H., Yasukawa, K., and Inouye, K. (2011) Thermodynamic analysis of ionizable groups involved in the catalytic mechanism of human matrix metalloproteinase 7 (MMP-7). *Biochim. Biophys. Acta* **1814**, 1940–1946
35. Bastock, J. A., Webb, M., and Grasby, J. A. (2007) The pH dependence of the *Escherichia coli* RNase HII-catalysed reaction suggests that an active site carboxylate group participates directly in catalysis. *J. Mol. Biol.* **368**, 421–433