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Improving the thermal stability of avian myeloblastosis virus reverse transcriptase α subunit by site-directed mutagenesis

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Abbreviations: AMV, avian myeloblastosis virus; MMLV, Moloney murine leukaemia virus; RT, reverse transcriptase; T/P, template-primer

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Abstract

Avian myeloblastosis virus reverse transcriptase (AMV RT) is a heterodimer consisting of a 63-kDa α subunit and a 95 k-Da β subunit. Moloney murine leukaemia virus reverse transcriptase (MMLV RT) is a 75-kDa monomer. These two RTs are the most extensively used as a tool for conversion of RNA to DNA. We previously developed several mutations that increase the thermostability of MMLV RT and generated a highly stable MMLV RT variant E286R/E302K/L435R/D524A by combining three of them (Glu286 \rightarrow Arg, Glu302 \rightarrow Lys, and Leu435 \rightarrow Arg) and the mutation to abolish RNase H activity (Asp524 \rightarrow Ala) (Yasukawa et al. (2010) J Biotechnol 150:299-306). In this study, to generate a highly stable AMV RT variant, we introduced the triple mutation of Val238 \rightarrow Arg, Leu388 \rightarrow Arg, and Asp450 \rightarrow Ala into AMV RT α subunit, and the resulted variant V238R/L388R/D450A was expressed in insect cells and purified. The temperatures reducing initial activity by 50% in 10-min incubation of the variant with or without template primer (T/P), poly(rA)-p(dT)₁₅, were 50°C, higher than for the wild-type AMV RT α subunit (WT) (44°C). The highest temperature at which the variant exhibited cDNA synthesis activity was 64°C, higher than for WT (60°C). These results indicate that highly stable AMV RT α subunit was generated by the same mutation strategy as applied to MMLV RT, that positive charges are introduced into RT at positions that have been implicated to interact with T/P by site-directed mutagenesis.

1. Introduction

Reverse transcriptase (RT) [EC 2.7.7.49] has RNA- and DNA-dependent DNA polymerase and RNase H activities, responsible for viral genome replication. Avian myeloblastosis virus reverse transcriptase (AMV RT) and Moloney murine leukaemia virus reverse transcriptase (MMLV RT) have high catalytic activity and fidelity in DNA synthesis. Hence these two RTs are the most extensively used in cDNA synthesis (Kimmel and Berger, 1987) and RNA-specific amplification (Kievits et al. 1991; Ishiguro et al. 2003). AMV RT is a heterodimer consisting of a 63-kDa α subunit and a 95-kDa β subunit. The β subunit comprises the fingers, palm, thumb, connection, RNase H, and intergase domains. The α subunit is a proteolytic cleavage product of the β subunit, and lacks the integrase domain. MMLV RT is a 75-kDa monomer comprising the same five domains as those of AMV RT α subunit. In AMV RT and MMLV RT, the active site of the DNA polymerase reaction resides in the fingers/palm/thumb domain, while that of RNase H reaction lies in the RNase H domain.

For cDNA synthesis and RNA-specific amplification procedures, an elevated reaction temperature is highly desirable because it reduces RNA secondary structure and nonspecific binding of the primer. However, RT is not thermally stable. The temperatures at which the initial reverse transcriptase activities of AMV RT and MMLV RT are reduced by 50% during a 10-min incubation, T_{50} , were 47 and 44°C, respectively, in the absence of a template-primer (T/P) and 52 and 47°C, respectively, in the presence of 28 μ M T/P (Yasukawa et al. 2008). As a result, reaction with RT has been performed at low temperatures such as 45-50°C for cDNA synthesis (Gerard, 1998) and 41-43°C for

RNA-specific amplification (Kievits et al. 1991; Ishiguro et al. 2003; Yasukawa et al. 2010a). If the reaction can be performed at higher temperatures, design of primers becomes easier. Considering that RNA is stable up to 65°C, stabilization of RTs is an important subject for their wide-range practical use. Based on that T/P is negatively charged and our result that T_{50} of AMV RT and MMLV RT in the presence of T/P are higher than in its absence (Yasukawa et al. 2008), we hypothesized that the thermal stability of RT will increase with increases in its ability to bind with T/P. We introduced positive charges into MMLV RT by site-directed mutagenesis at positions that have been implicated in the interaction with T/P, and expressed 36 single variants in *Escherichia coli*. After characterizing their thermal stabilities, we combined the three highest stabilizing mutations (Glu286→Arg, Glu302→Lys, and Leu435→Arg) and the mutation that has been known to abolish the RNase H activity and increase stability, Asp524→Ala. The T_{50} in the presence of T/P of the resulted variant, E286R/E302K/L435R/D524A, was 56°C, higher by 10°C than that of the wild-type enzyme. The highest temperature at which the variant exhibited cDNA synthesis activity was 60°C, higher by 6°C than for the wild-type enzyme (Yasukawa et al. 2010c).

Considering that the sequence homology between AMV RT and MMLV RT is 23%, we hypothesized that the results obtained in our study of MMLV RT (Yasukawa et al. 2010c) is applicable to stabilize AMV RT. Sequence comparison reveals that Glu286, Glu302, Leu435, and Asp524 of MMLV RT correspond to Val238, Lys254, Leu388, and Asp450, respectively, of AMV RT (Fig. 1). One of the problems is that unlike recombinant MMLV RT (Chen et al. 2009; Yasukawa et al. 2010c), recombinant AMV RT has barely been expressed in soluble fractions in *E. coli* (unpublished results). We previously expressed recombinant wild-type AMV RT α subunit (WT) in insect cells and purified it from the cells (Konishi et al. 2011).

In this study, we prepared the AMV RT α subunit variant V238R/L388R/D450A and compared its thermal stability with that of WT.

2. Materials and methods

2.1. Expression in insect cells and purification of AMV RT α subunit

Expression in insect cells and purification of AMV RT α subunit was performed as described previously (Konishi et al. 2011). Briefly, *E. coli* DH10Bac (Life Technologies Japan Ltd, Tokyo, Japan), which contains a baculovirus shuttle vector (bacmid) and a helper plasmid, was transformed with pFastBac1-ART α WT or pFastBac1-ART α AM4, which are the expression plasmids for the C-terminally (His)₆-tagged wild-type AMV RT α subunit (WT) and V238R/L388R/D450A, respectively. The recombinant bacmid was prepared from the transformed DH10Bac cells and transfected into Sf9 insect cells. For expression of the α subunit, 100 ml of Sf9 cells at a density of 1×10^6 cells/ml was infected with the supernatant containing the recombinant baculovirus and cultivated for 120 h at 28°C. The α subunit was purified from the cells by successive procedures of ammonium sulfate fractionation, anion-exchange chromatography, and Ni²⁺ affinity chromatography and stored at -80°C.

2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in a 10% polyacrylamide gel under reducing conditions.

Proteins were reduced by treatment with 2.5% (v/v) of 2-mercaptoethanol at 100°C for 10 min, and then applied onto the gel. A constant current of 40 mA was applied for 40 min. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250. The molecular mass marker kit consisting of rabbit muscle phosphorylase B (97.2 kDa), bovine serum albumin (66.4 kDa), hen egg white ovalbumin (44.3 kDa), and bovine carbonic anhydrase (29.0 kDa) was a product of Takara Bio Inc (Otsu, Japan).

2.3. Measurement of AMV RT α subunit activity to incorporate dTTP into poly(rA)-p(dT)₁₅

The activity of the α subunit to incorporate dTTP into poly(rA)-p(dT)₁₅ was performed as described previously (Yasukawa et al. 2010b). Briefly, poly(rA)-p(dT)₁₅ was prepared from p(dT)₁₅ (Life Technologies Japan Ltd) and poly(rA) (GE Healthcare, Buckinghamshire, UK). [³H]dTTP solution (1.85 Bq/pmol) was prepared from [³H]dTTP (1.52 TBq/mmol) (GE Healthcare) and dTTP (GE Healthcare). The reaction was carried out in 25 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM DTT, 5.0 mM MgCl₂, 25 μ M poly(rA)-p(dT)₁₅ (this concentration expressed as that of p(dT)₁₅), 0.4 mM [³H]dTTP, and 10% (v/v) samples at 37°C. An aliquot (20 μ l) was taken from the reaction mixture at a predetermined time and immediately spotted onto the glass filter GF/C 2.5 cm (Whatman, Middlesex, UK). The amounts of [³H]dTTP incorporated was counted, and the initial reaction rate was determined. The kinetic parameters, Michaelis constant (K_m) and molecular activity (k_{cat}), were determined with Kaleida Graph Version 3.5 (Synergy Software, Essex, VT).

2.4. Irreversible thermal inactivation of AMV RT α subunit

AMV RT α subunit (30 nM) in 10 mM potassium phosphate (pH 7.6), 2.0 mM DTT, 0.2% (v/v) Triton X-100, and 10% (v/v) glycerol was incubated in the presence or absence of 28 μ M poly(rA)-p(dT)₁₅ at a range of temperatures from 42 to 50°C for 0-30 min followed by incubation on ice for 30-60 min. The remaining RT activity to incorporate dTTP into poly(rA)-p(dT)₁₅ was determined at 37°C as described above.

2.5. Thermodynamic analysis of irreversible thermal inactivation of AMV RT α subunit

On the assumption that the thermal inactivation reaction of RT is irreversible and consists of only one step, the first-order rate constant of the thermal inactivation (k_{obs}) was evaluated by plotting logarithmic values of the relative activity, the ratio of the initial reaction rate with incubation for the indicated durations to that without incubation, against the time of thermal treatment according to Eq. 1.

$$\ln B = A - k_{\text{obs}}t \quad (1)$$

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 thermal treatment ($=t$) to that without the treatment. The activation energy for the thermal inactivation (E_a) was determined from Arrhenius plot according to Eq. 2.

$$\ln(k_{\text{obs}}) = A - (E_a/R)(1/T) \quad (2)$$

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 kelvin, respectively. T_{50} was estimated by Arrhenius plot as the temperature at which the k_{obs} value gives the remaining activity of 50% at 10 min according to Eq. 2.

2.6. Measurement of AMV RT α subunit activity for cDNA synthesis

The activity of the α subunit for cDNA synthesis was measured as described previously (Yasukawa et al. 2010a). Briefly, standard RNA, which was an RNA of 1014-nucleotides corresponding to DNA sequence 8353-9366 of the *cesA* gene of *Bacillus cereus* (GenBank accession number DQ360825), was prepared by an *in vitro* transcription. The reaction (20 μl) was carried out in 25 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM DTT, 0.1 mM dNTP, 0.5 μM R12 primer (5'-TGTGGAATTGTGAGCGGTGTCGCAATCACCGTAACACGACGTAG-3'), 0.08 pg/ μl standard RNA, 0.05 $\mu\text{g}/\mu\text{l}$ *E. coli* RNA, and 10 nM AMV RT α subunit at a range of temperatures from 46 to 66°C for 30 min and stopped by heating at 95°C for 5 min. The PCR reaction (30 μl) was then carried out in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl_2 , 0.5 μM F5 primer (5'-TGCGCGCAAATGGGTATCAC-3'), 0.5 μM RV primer (5'-TGTGGAATTGTGAGCGG-3'), 0.1 mM dNTP, 10% (v/v) product of the reverse transcriptase reaction, and 0.05 U/ μl recombinant *Taq* polymerase (Toyobo, Osaka, Japan). The cycling parameters were 95°C for 30 s, followed by 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The amplified products were separated on 1.0% agarose gels and stained with ethidium bromide (1 $\mu\text{g}/\text{ml}$).

3. Results

3.1. Production of AMV RT α subunit

The wild-type AMV RT α subunit (WT) and V238R/L388R/D450A were expressed in insect cells and purified from the cells. Upon SDS-PAGE under reducing condition, both enzyme preparations yielded a single band with a molecular mass of 63 kDa, while MMLV RT, which was expressed in *E. coli* and purified from the cells (Yasukawa et al. 2008), yielded a single band at 75 kDa (Fig. 2).

3.2. Steady-state kinetic analysis of AMV RT α subunit

The kinetic parameters of the α subunits in the incorporation of dTTP into poly(rA)-p(dT)₁₅ (T/P) were examined. Figure 3 shows the initial reaction rates for 10 nM AMV RT α subunit at various T/P concentrations (0-25 μ M) at 37°C. A saturated profile of the Michaelis-Menten kinetics was obtained. The K_m and k_{cat} values were $18.8 \pm 4.4 \mu$ M and $7.4 \pm 0.9 \text{ s}^{-1}$, respectively, for WT, and $7.0 \pm 0.9 \mu$ M and $7.7 \pm 0.4 \text{ s}^{-1}$, respectively, for V238R/L388R/D450A, indicating that the K_m value of V238R/L388R/D450A was 40% of that of WT, while their k_{cat} values were almost the same. This suggests that V238R/L388R/D450A has higher affinity for T/P than WT.

3.3. Irreversible thermal inactivation of AMV RT α subunit

The remaining reverse transcriptase activities of the AMV RT α subunits were determined at 37°C after thermal treatment (42-46°C for WT and 46-50°C for V238R/L388R/D450A) in the presence and the absence of T/P. Figure 4A shows the results with the thermal treatment at 46°C (data not shown for the results with the treatment at other temperatures). The linear relationship between the natural logarithm of the remaining activity and the incubation time indicated that the inactivation followed pseudo-first-order kinetics. The relative activity of WT decreased to less than 10% at 25 min, while that of V238R/L388R/D450A hardly decreased at 0-30 min, indicating that V238R/L388R/D450A is more stable than WT.

Figure 4B shows an Arrhenius plot of k_{obs} of the thermal inactivation of the α subunits in the presence and the absence of T/P. The natural logarithm of k_{obs} and $1/T$ showed a linear relationship. The temperatures required to reduce initial activity by 50% over a 10-min incubation (T_{50}) were calculated by this plot. The T_{50} values without and with T/P of WT were 44.3 and 44.1°C, respectively, and those of V238R/L388R/D450A were 50.1 and 49.3°C, respectively, indicating that V238R/L388R/D450A is more stable than WT and that the α subunits were not stabilized by T/P. The activation energies (E_a) of thermal inactivation of the α subunits were calculated from the slope of this plot. The E_a values without and with T/P of WT were 194 ± 43 , 244 ± 40 kJ mol⁻¹, respectively, and those of V238R/L388R/D450A were 240 ± 23 , and 298 ± 0 kJ mol⁻¹, respectively, indicating that all E_a values were similar.

3.4. AMV RT α subunit-catalyzed cDNA synthesis

The activities of the α subunits for cDNA synthesis reaction were examined. cDNA synthesis reaction carried out with WT and V238R/L388R/D450A at 44-66°C, followed by PCR of the cDNA synthesis reaction products. Figure 5 shows the results with agarose gel electrophoresis of the PCR products. The highest temperatures at which the amplified product with the expected size of 601 bp was detected were 60°C for WT and 64°C for V238R/L388R/D450A, indicating that V238R/L388R/D450A is more stable than WT in cDNA synthesis.

4. Discussion

4.1. Increase in RT stability by introducing positive charges by site-directed mutagenesis

In the previous study of MMLV RT, the quadruple mutation of Glu286→Arg, Glu302→Lys, Leu435→Arg, and Asp524→Ala increased the highest temperature at which cDNA synthesis reaction occurred by 6°C (from 54 to 60°C), and decreased the K_m value for T/P to 40% of that without the mutation in the incorporation of dTTP into T/P (Yasukawa et al. 2010c). In this study of AMV RT α subunit, the triple mutation of Val238→Arg, Leu388→Arg, and Asp450→Ala, which corresponds to the quadruple mutation introduced into MMLV RT, increased the highest temperature at which cDNA synthesis reaction occurred by 4°C (from 60 to 64°C) (Fig. 5), and decreased the K_m value for T/P to 40% of that without the mutation in the incorporation of dTTP into T/P (Fig. 3). These results suggest that introduction of positive charges into the enzyme molecule at positions that have been implicated in the interaction with T/P by site-directed mutagenesis is effective to stabilize

not only MMLV RT and AMV RT but also other RTs.

It is known that the mutations to abolish RNase H activity of RT increase its thermostability although its mechanism is not clarified (Gerard et al. 2002; Mizuno et al. 2010). Except for such mutations, the stabilizing mutations so far reported in MMLV RT are Glu69→Lys, Glu302→Arg, Trp313→Phe, Leu435→Gly, and Asn454→Lys (Arezi and Hogrefe, 2009). Interestingly, these mutations were developed by random mutagenesis, but the multiple variant E69K/E302R/W313F/L435G/N454K has lower K_m value for T/P than the wild-type MMLV RT (Arezi and Hogrefe, 2009). In HIV-1 RT, it was recently reported that the HIV-1 group O RT is more stable than HIV-1 group M (major-typed) RT and is as stable as MMLV RT (Álvarez et al. 2009; Barrioluengo et al. 2011). The sequence homology between these two HIV-1 RTs is 21%, and the amino acid residues essential for the difference in stability have not been identified.

Another approach to develop thermostable reverse transcriptase is to generate RNA-dependent DNA polymerase activity in thermostable DNA-dependent DNA polymerase by a genetic engineering technique (Schönbrunner et al. 2006). Recently, double mutation of Leu329→Ala and Gln384→Ala in family A DNA polymerase from *Thermotoga petrophila* K4 generated RNA-dependent DNA polymerase activity although the magnitude of the RNA-dependent DNA polymerase activity was smaller than that of MMLV RT (Sano et al. 2012). In that study, the mutations were introduced at positions that have been implicated in the discrimination of DNA and RNA by steric interference with the 2'-hydroxyl group of ribose (Sano et al. 2012).

4.2. Effects of T/P on the stabilization of RT

The triple mutation of Val238→Arg, Leu388→Arg, and Asp450→Ala increased the T_{50} of AMV RT α subunit from 54 to 60°C both in the presence and absence of T/P (Fig. 4). This means that T/P does not stabilize the wild-type AMV RT α subunit or its thermostable variant V238R/L388R/D450A. This is in contrast to that T/P stabilizes native AMV RT consisting of α and β subunits and native MMLV RT (Gerard et al. 2002; Yasukawa et al. 2008; Konishi et al. 2011).

Integrase is the enzyme that is required for the integration of the viral DNA synthesized in the host cell from the viral RNA genome into the host cell DNA genome. In AMV RT, the β subunit has the integrase domain, but the α subunit does not. Our results suggest that the integrase domain is required for the stabilization of AMV RT by T/P. Without the integrase domain, introduced positive charges increase the affinity of AMV RT for T/P, but do not result in the stabilization by T/P. We hypothesize that unlike native AMV RT, introduced positive charges make AMV RT α subunit thermally resistant only by increasing intrinsic enzyme stability. This hypothesis should be explored by characterizing recombinant wild-type AMV RT heterodimer consisting of α and β subunits and its variant with the triple mutation of Val238→Arg, Leu388→Arg, and Asp450→Ala in the α and/or β subunits. It should be noted that the expression level of the β subunit in insect cells is markedly lower than that of the α subunit (unpublished data), and thus optimization of preparation conditions is currently under way.

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Figure legends

Fig. 1. Structure of MMLV RT and AMV RT α subunit. Amino acid numberings of MMLV RT (Thr24-Leu671) and AMV RT α subunit (Thr1-Tyr572) are according to GenBank accession codes J02255 and FJ041197, respectively. Asterisks show homologous amino acid residues. The amino acid residues to be mutated are underlined.

Fig. 2. SDS-PAGE under reducing conditions. Coomassie Brilliant Blue-stained 10% SDS-polyacrylamide gel is shown. Lane 1, molecular-mass marker; lane 2, wild-type AMV α subunit (WT); lane 3, AMV RT α subunit variant V238R/L388R/D450A; lane 4, wild-type MMLV RT.

Fig. 3. Dependence on substrate concentration of the initial reaction rate of AMV RT α subunit-catalyzed incorporation of dTTP into poly(rA)-p(dT)₁₅ at 37°C. The initial concentrations of AMV RT α subunit and dTTP were 10 nM and 200 μ M, respectively. Solid lines represent the best fit of the Michaelis-Menten equation with the non-linear least squares method. Symbols for the enzymes: WT, open circle; V238R/L388R/D450A, open triangle. The average of triplicate determination is shown.

Fig. 4. Irreversible thermal inactivation of AMV RT α subunit. AMV RT α subunit at 30 nM was incubated at 42-50°C in the presence or absence of poly(rA)-p(dT)₁₅ (T/P) (28 μ M) for the indicated durations. Then, the reverse transcription reaction was carried out at 37°C. The relative activity of AMV RT α subunit was defined as the ratio of the initial reaction rate

with incubation for the indicated durations to that without incubation [14.8 nM s⁻¹ for WT (3 nM) without T/P, 16.2 nM s⁻¹ for WT (3 nM) with T/P, 18.9 nM s⁻¹ for V238R/L388R/D450A (3 nM) without T/P, 19.3 nM s⁻¹ for V238R/L388R/D450A (3 nM) with T/P]. (A) Thermal inactivation at 46°C. The first-order rate constant of the thermal inactivation (k_{obs}) of RT was estimated from the slope: WT without T/P (open circle), $1.9 \times 10^{-3} \text{ s}^{-1}$; WT with T/P (closed circle), $1.7 \times 10^{-3} \text{ s}^{-1}$; V238R/L388R/D450A without T/P (open triangle), $3.6 \times 10^{-4} \text{ s}^{-1}$; V238R/L388R/D450A with T/P (closed triangle), $3.5 \times 10^{-4} \text{ s}^{-1}$. (B) Arrhenius plot of k_{obs} values. The activation energy of thermal inactivation (E_a) of RT with or without T/P was calculated from the slope: WT without T/P (open circle), $194 \pm 43 \text{ kJ mol}^{-1}$; WT with T/P (closed circle), $244 \pm 40 \text{ kJ mol}^{-1}$; V238R/L388R/D450A without T/P (open triangle), $240 \pm 23 \text{ kJ mol}^{-1}$; V238R/L388R/D450A with T/P (closed triangle), $298 \pm 0 \text{ kJ mol}^{-1}$.

Fig. 5. Dependence on reaction temperature of cDNA synthesis by AMV RT α subunit. cDNA synthesis was carried out with 1.6 pg *cesA* RNA, 0.2 μM RV-R12 primer, and 10 nM AMV RT α subunit at 44-66°C. PCR was carried out with a primer combination of RV and F5. Amplified products were applied to 1.0% agarose gel followed by staining with ethidium bromide (1 $\mu\text{g/ml}$). The arrow indicates the expected size (601 bp) of the amplified products.

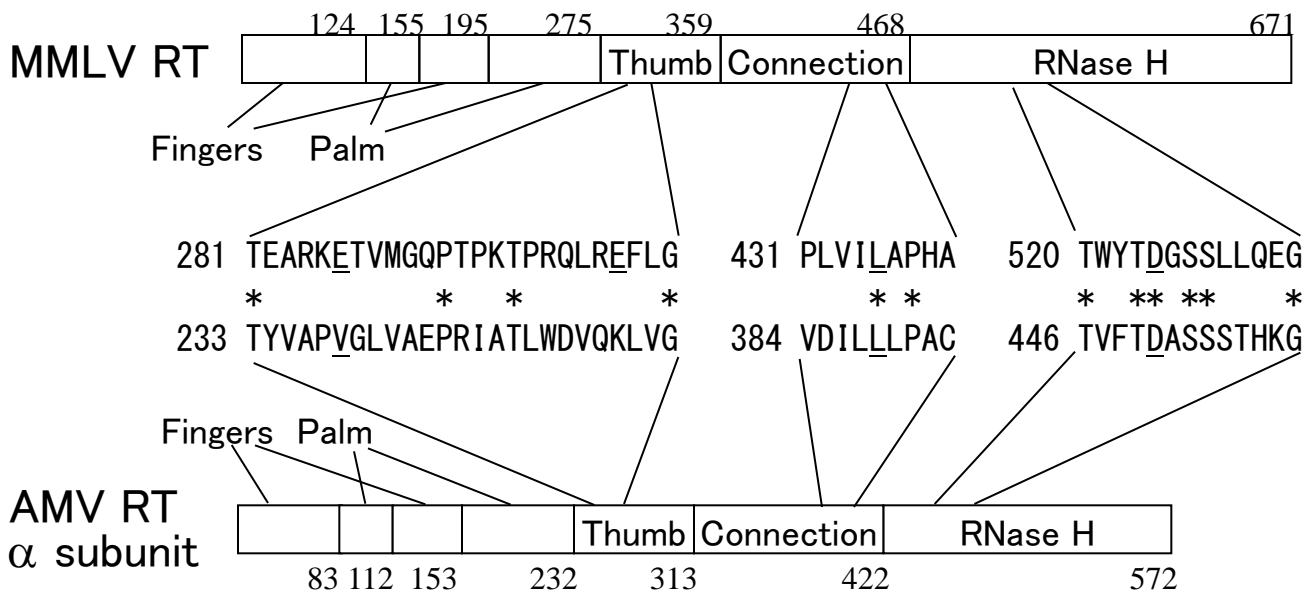


Fig. 1

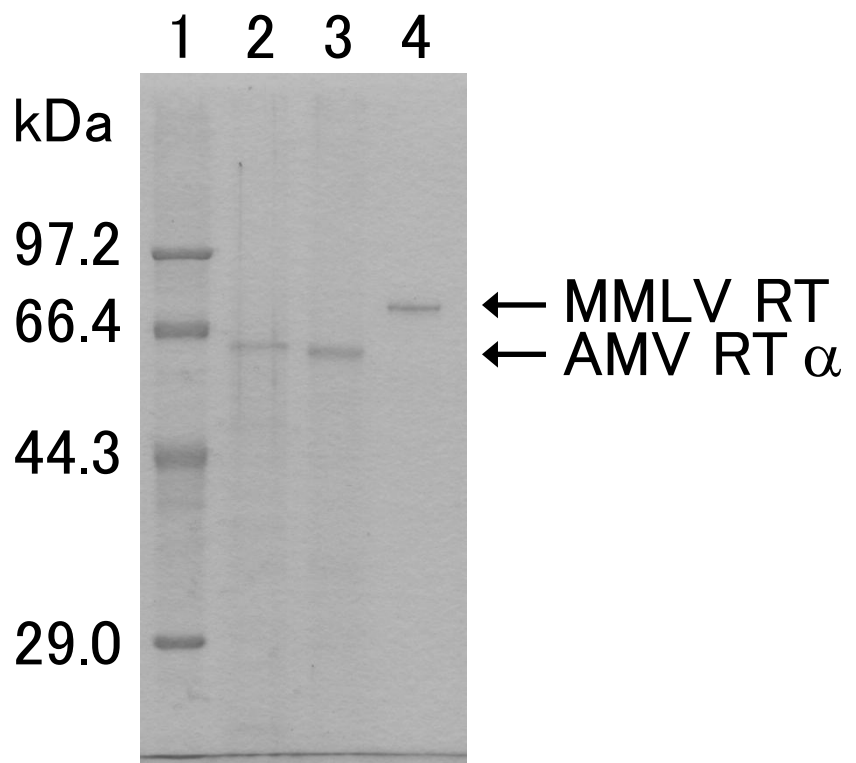


Fig. 2

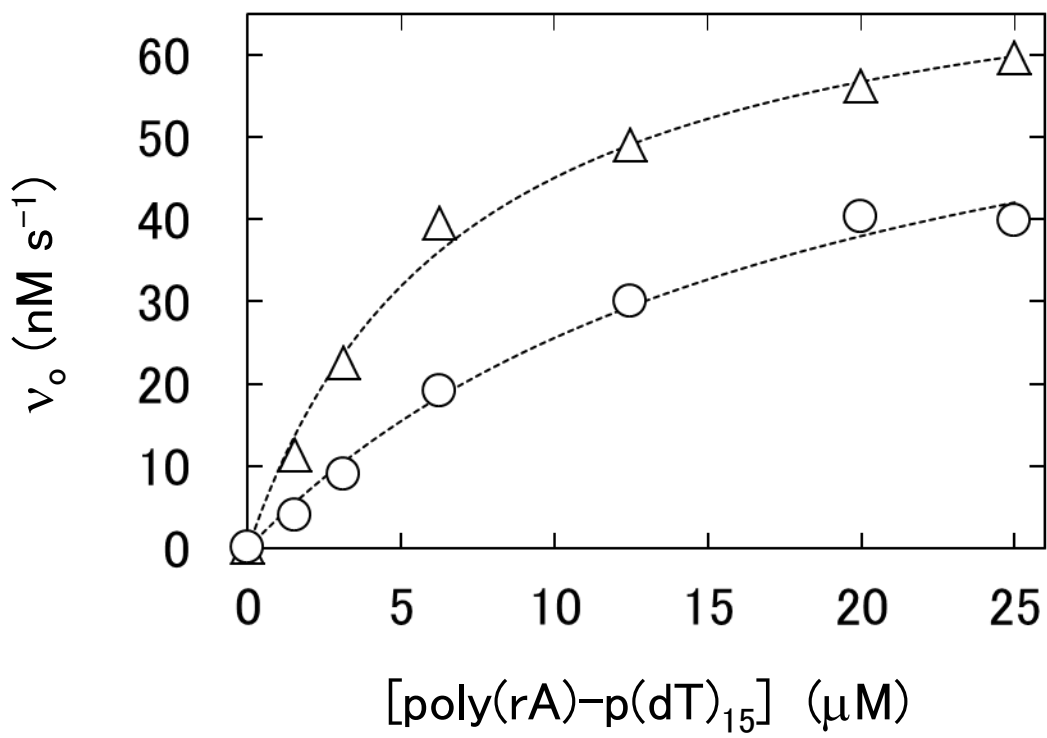
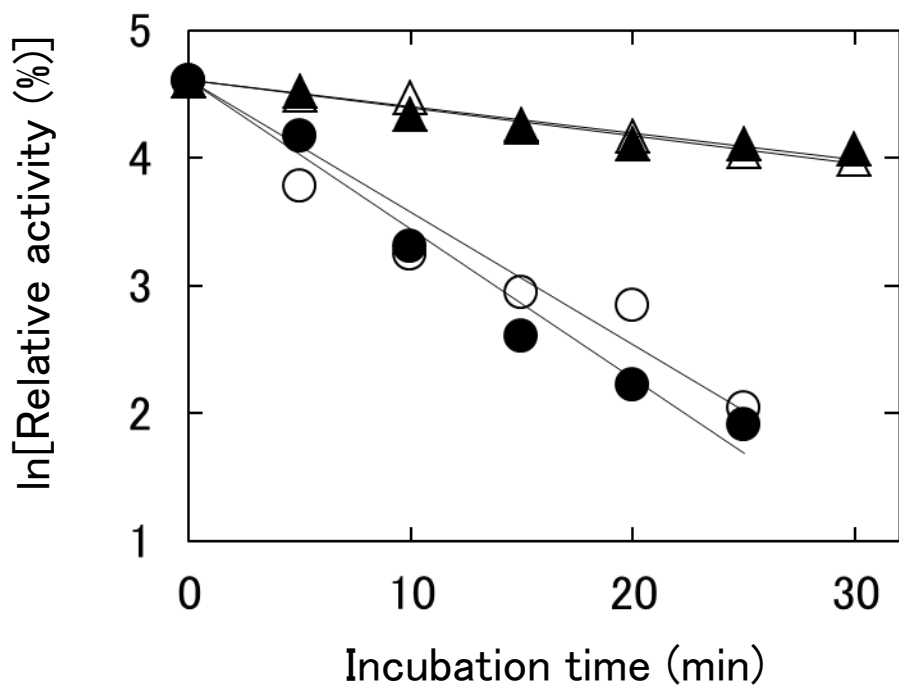


Fig. 3

A



B

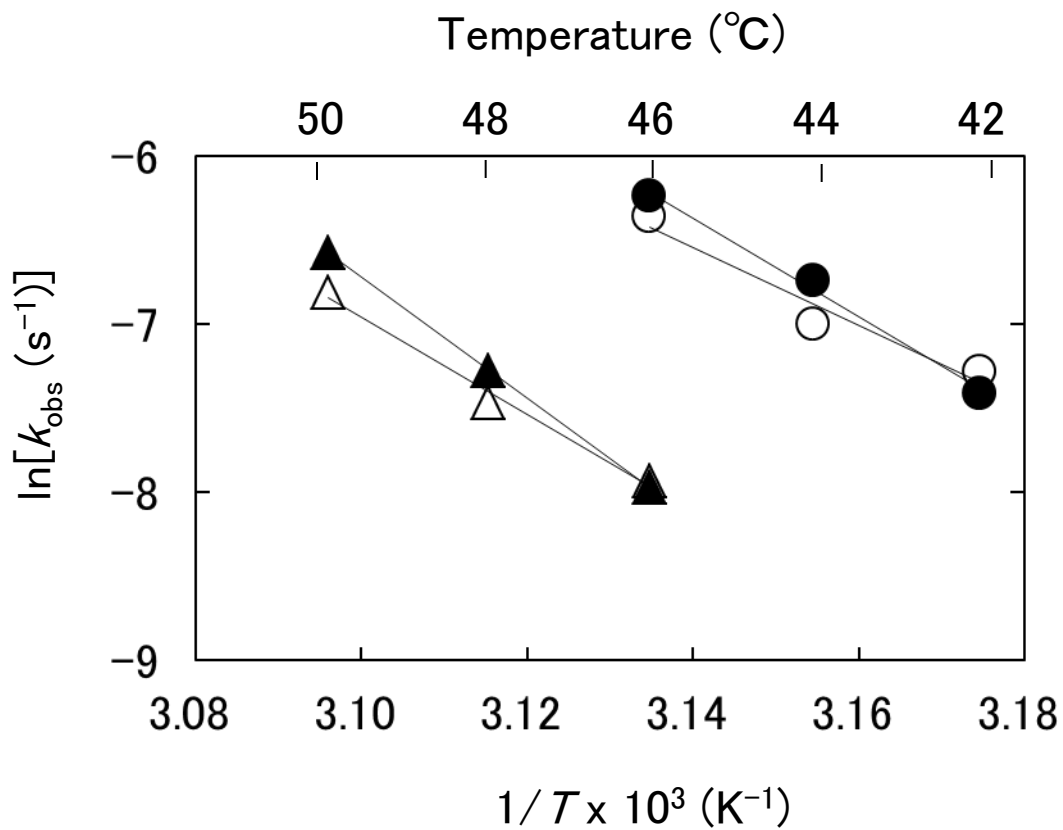


Fig. 4

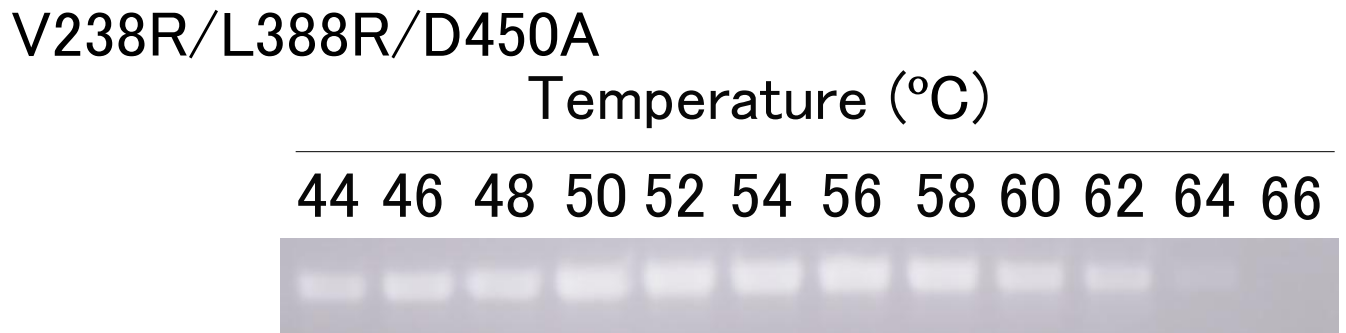
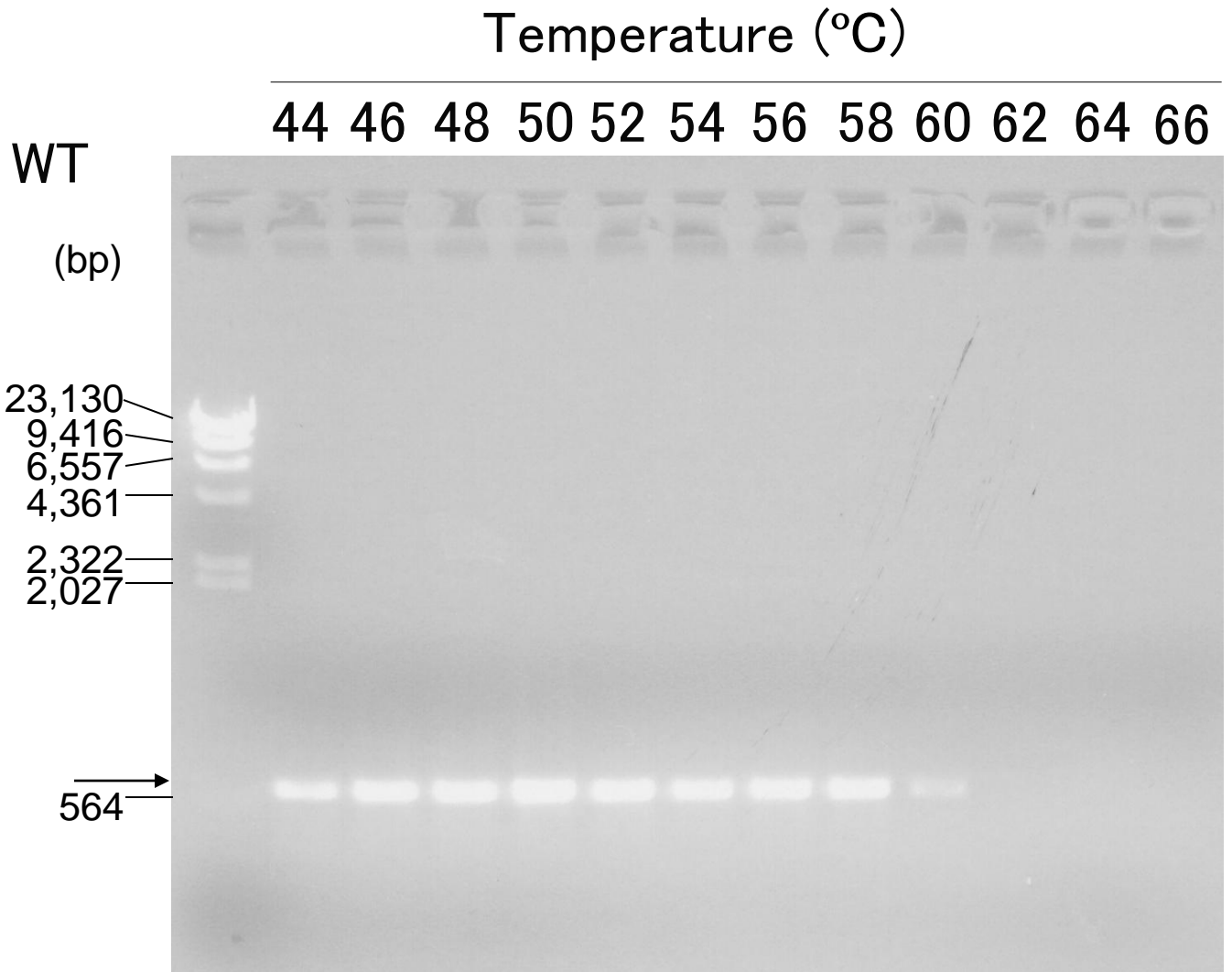


Fig. 5