Walleye dermal sarcoma virus reverse transcriptase is temperature sensitive

Sharon K. Fodor† and Volker M. Vogt

Department of Molecular Biology and Genetics, Biotechnology Building, Cornell University, Ithaca, NY 14853, USA

Walleye dermal sarcoma virus (WDSV) is a piscine retrovirus that replicates naturally in fish at temperatures near 4 °C. The reverse transcriptase (RT) protein from virus particles isolated from walleye tumours was purified and biochemically characterized. Like the RT of the distantly related murine leukaemia virus, WDSV RT sediments as a monomer in the absence of template. It exhibits a $K_m$ of 22 μM for TTP in an assay with poly(rA) as a template and oligo(dT) as a primer. The enzyme is rapidly inactivated at temperatures greater than 15 °C. The ratio of RT activity at 15 °C to that at 4 °C is similar for WDSV and recombinant human immunodeficiency virus type 1, suggesting that, at least with this template, the fish enzyme is not specially adapted to function more efficiently in the cold.

Walleye dermal sarcoma virus (WDSV) is a piscine retrovirus associated with skin tumours in walleyes (Bowser et al., 1988). WDSV is the prototype of the Epsilonretrovirus genus, which includes the closely related walleye epidermal hyperplasia viruses types 1 and 2 (WEHV-1 and -2) (Holzschu et al., 1995; LaPierre et al., 1999; van Regenmortel et al., 2000). Based on sequence similarity in the reverse transcriptase (RT) gene, the walleye viruses are most closely related to the mammalian C-type viruses or gammaretroviruses (van Regenmortel et al., 2000). WDSV and murine leukaemia virus (MuLV) share 45% sequence identity within the polymerase domain of RT. In addition, these viruses are similar in that they both utilize termination suppression as the mechanism for production of the Gag–Pol polyprotein and both have only a single Cys–His motif in the non-coding domain of Gag. However, MuLV and the other members of this genus are simple retroviruses, while WDSV is a complex retrovirus, exhibiting differential mRNA splicing and containing three open reading frames (ORFs) in addition to gag, pol and env. One of these ORFs encodes a cyclin D homologue believed to be responsible for the oncogenic potential of the virus (Holzschu et al., 1995; Lairmore et al., 2000; LaPierre et al., 1998; Quackenbush et al., 1997).

The walleye, the host species for WDSV, is a poikilotherm and its body temperature depends on the temperature of the environment. WDSV-induced tumours exhibit seasonality: tumours develop on the fish in the fall, persist through the winter and subsequently regress and fall off in the spring. The fall tumours do not contain infectious virus, and viral genomic RNA and proteins are not detectable, although subgenomic RNA is present. In contrast, spring tumours contain large quantities of virus (Martineau et al., 1992). Infection of walleyes is believed to take place during the spring spawning season when the fish are in close contact, at which time the water temperature is approximately 4 °C. For infection to occur at this time, RT, a DNA polymerase that reverse transcribes the viral RNA genome to yield a double-stranded DNA copy, must be able to synthesize proviral DNA at low temperatures. The goal of the present study was to carry out an initial biochemical characterization of WDSV RT and to investigate the effect of temperature on the enzyme.

The starting material for RT purification was WDSV from walleye tumours. The virus was isolated from mashed tumours submitted to differential centrifugation, followed by isopycnic centrifugation in sucrose gradients (Holzschu et al., 1995; Martineau et al., 1991). RT was purified from virions by affinity chromatography using either poly(U)–Sepharose or heparin–Sepharose followed by rate-zonal sedimentation through a 20–40% glycerol gradient using the protocol of Grandgenett et al. (1978). Purification of RT using either heparin–Sepharose or poly(U)–Sepharose yielded similar results, as determined by SDS–PAGE analysis of chromatography fractions (data not shown). RT activity was monitored using a poly(rA)/oligo(dT) assay (Martineau et al., 1991; Telesnitsky et al., 1995). Purified RT was dialysed into storage buffer (50% glycerol, 50 mM Tris, pH 7.5, 0.1 mM EDTA, 2 mM DTT, 0.02% NP-40 and 100 mM KCl). The concentration of heparin-purified RT polypeptide was estimated,
by silver staining, to be 10 ng/µl (data not shown). The final purity was estimated to be 30–50% (Fig. 1A) and this enzyme preparation was used in all subsequent enzymatic assays. Limited quantities of virus available as starting material precluded further purification steps.

RT is composed of a polymerase domain that can use either DNA or RNA as a template and an RNase H domain that degrades the RNA strand from an RNA–DNA duplex (Johnson et al., 1986; Tanese & Goff, 1988). The subunit composition of RT varies with the virus genus. MuLV RT is a monomer in solution, containing both the polymerase and RNase H domains, but the enzyme may dimerize when bound to template (Telesnitsky & Goff, 1993). Human immunodeficiency virus type 1 (HIV-1) RT is a heterodimer consisting of a p66 subunit, containing the polymerase and RNase H domains, and a p51 subunit, containing only the polymerase domain (Hansen et al., 1988; Hostomsky et al., 1991; Le Grice & Gruninger-Leitch, 1990; Mizrahi, 1989; Mizrahi et al., 1989; Prasad & Goff, 1989; Veronese et al., 1986). The RT of avian sarcoma and leukaemia virus (ASLV) is a different type of heterodimer, with one subunit containing the polymerase and RNase H domains and the other subunit containing the polymerase, RNase H and integrase domains (Golomb & Grandgenett, 1979; Grandgenett et al., 1985).

The predicted molecular mass of the WDSV RT polypeptide, based on protease cleavage sites in Pol, is 72.5 kDa (Fodor & Vogt, 2002). The polymerase domain has a predicted molecular mass of approximately 50 kDa, the RNase H domain is approximately 20 kDa and the integrase domain is approximately 40 kDa. Thus, an RT heterodimer should be about 120 or 180 kDa if it had a subunit composition like that of HIV-1 or ASLV, respectively, while an RT monomer would be about 70 kDa if it were similar to MuLV. To experimentally address the subunit composition of WDSV RT, we compared the sedimentation of RT in a glycerol gradient with the sedimentation of known standards. Rate-zonal sedimentation of RT eluted from poly(U)–Sepharose was performed in parallel with the markers phosphorylase B (97 kDa), BSA (69 kDa), ovalbumin (46 kDa) and carbonic anhydrase (30 kDa). The activity peak coincided with the migration of the BSA marker (Fig. 1B, lane 11). Since RTs are approximately globular, we interpret this result to mean that WDSV RT is a monomer in solution, similar to MuLV RT.

In its natural environment, WDSV infects fish at a temperature near 4 °C, raising the possibility that WDSV RT is specially adapted to function at low temperature. To address this question, we analysed RT activity at 4, 16, 25 and 37 °C. Purified HIV-1 RT obtained from Escherichia coli cells over-expressing the His-tagged p66 subunit was used as a control (Le Grice et al., 1995). HIV-1 p66 and WDSV RT exhibited similar rates of TTP incorporation at 4 °C, normalized for amount of enzyme, and both enzymes demonstrated increasing rates of activity with increasing temperature (Fig. 2A). However, a comparison of the specific activity of both polymerases at temperatures greater than 4 °C demonstrated that the specific activity of WDSV was lower than that of HIV-1. Unlike HIV-1, WDSV showed maximum activity at 25 °C, while activity was negligible at 37 °C, suggesting that the
**Fig. 2.** WDSV RT is temperature sensitive. (A) RT activity as a function of time. RT activity was measured at 37, 25, 16 and 4 °C for up to 2 h. Assays contained 100 nM WDSV RT or HIV-1 p66 and 50 mM Tris, pH 8.0, 60 mM KCl, 0-6 mM MgCl₂, 2 mM DTT, 0-2% Triton X-100, 20 µg/ml poly(rA), 5 µg/ml oligo(dT)₁₂₋₁₈, 10 µCi/ml [³²P]dTTP and 10 µM dTTP. Incorporated dTTP was bound to DEAE paper (DE-81, Whatman) and quantified in a scintillation counter. WDSV incubated at 37 °C (+), 25 °C (U), 16 °C (E) and 4 °C ( BELOW); HIV-1 incubated at 37 °C (*), 25 °C (V), 16 °C (D) and 4 °C ( ). (B) RT activity as a function of temperature. Reactions were incubated for 60 min at 37, 25, 16 and 4 °C. (C) Heat inactivation of WDSV RT. Reaction mixtures were preincubated, in the absence of nucleotides, at 37, 30, 25, 16 or 4 °C for the times indicated. RT activity was then assayed at room temperature for 15 min. Preincubation at 0 °C was assigned 100% activity. °, 37 °C; •, 30 °C; ●, 25 °C; ▲, 16 °C; ▼, 4 °C.

**Fig. 3.** pH, divalent cation and salt requirements for RT activity. (A) WDSV RT activity was measured at pH 6-12. Reactions contained 200 mM potassium phosphate (pH 6-0), 200 mM Tris (pH 7 or 8) or 100 mM glycine (pH 9, 10, 11 or 12). (B) Salt requirement was determined at 50, 100, 150, 200, 250 and 300 mM KCl. (C) Divalent cation requirement was determined at 2, 4, 6, 8 and 10 mM MgCl₂ (▲) or MnCl₂ (●). Except where otherwise noted, RT assays contained 20 mM Tris, pH 8-0, 50 mM KCl, 2 mM MgCl₂, 10 mM DTT, 0-2% Triton X-100, 20 mg/ml poly(rA), 5 mg/ml oligo(dT)₁₂₋₁₈, 10 µCi/ml [³²P]dTTP and 10 µM dTTP. Reactions were incubated at 16 °C for 30 min and stopped with EDTA. All values are reported as percentage activity, where 100% was set at the condition yielding maximum activity.
enzyme is thermosensitive. This apparent inactivation could represent instability of the protein because of purification or an inherent characteristic of the enzyme. To distinguish these possibilities, RT activity in crude lysates of WDSV virions was compared with the activity of purified enzyme (Fig. 2B). Thermosensitivity was found to be the same for both, suggesting that temperature inactivation was due to loss of stabilizing factors during purification.

Heat inactivation of RT was evaluated more precisely by preincubating enzyme with template at various temperatures and then determining the residual activity at room temperature (Fig. 2C). Following preincubation at 4 and 16 °C, activity was unaffected. However, 50% inactivation occurred in 35 min at 25 °C, in 10 min at 30 °C and in less than 10 min at 37 °C.

To further characterize WDSV RT, its requirements for pH, salt and divalent cation were determined in assays carried out at 16 °C. The enzyme showed activity over a broad pH range, with a maximum near pH 8 (Fig. 3A). The effect of ionic strength on RT activity was measured at 50, 100, 150, 200, 250 and 300 mM KCl (Fig. 3B). As reported for other retroviral RTs (Roth et al., 1985; Taube et al., 1998), increasing ionic strength inhibited the enzyme. WDSV RT activity was highest at 50 mM KCl and decreased with increasing KCl concentrations. Retroviral RTs require divalent cations for polymerization, with most RTs preferring Mg++. However, MuLV RT is distinct in that it displays maximal activity on a poly(rA) template in the presence of Mn++. These data clearly demonstrate that WDSV RT shows optimal activity in magnesium ions showed WDSV RT to be most active with Mg++, over a wide range of concentrations (Fig. 3C). In contrast, use of Mn++ as the divalent cation did not stimulate polymerization but rather inhibited activity with increasing concentration. It should be noted that the range of concentrations tested (2–10 mM) was above the optimal manganese concentration (0.5 mM) determined for MuLV (Roth et al., 1985; Verma, 1975). Activity assays in the presence of these divalent metal ions showed WDSV RT to be more active with Mg++, over a wide range of concentrations (Fig. 3C). In contrast, use of Mn++ as the divalent cation did not stimulate polymerization but rather inhibited activity with increasing concentration. It should be noted that the range of concentrations tested (2–10 mM) was above the optimal manganese concentration (0.5 mM) determined for MuLV (Roth et al., 1985; Verma, 1975). However, divalent cation preference of WDSV RT at a similar concentration (0.6 mM) was determined at room temperature using lysed virions (data not shown) and exhibited 1.5 times more activity in Mg++ compared to Mn++. In contrast, MuLV RT was 50 times more active in 0.5 mM Mn++ than in 0.5 mM Mg++ (Roth et al., 1985). These data clearly demonstrate that WDSV RT shows optimal activity in magnesium and is distinct from MuLV in this characteristic.

The $K_m$ was determined for WDSV RT in the poly(rA)/oligo(dT) assay using dTTP as the substrate. Initial velocities were measured at 16 °C with substrate concentrations of 5, 10, 25, 50 or 75 mM dTTP. The $K_m$ calculated from these data was 22 μM, which is in the range reported for other RTs (Hizi et al., 1991; Ricchetti & Buc, 1990; Taube et al., 1998). As a control, the $K_m$ determined in parallel for HIV-1 p66 was 5 μM. This value is consistent with the reported value of 5.9 μM (Hizi et al., 1991), which was determined for the p51/p66 heterodimer of HIV-1 rather than p66 homodimers used in this study. The turnover number calculated for WDSV RT at 16 °C was 0.3 s⁻¹. Assuming that the turnover rate is identical for all nucleotides and ignoring the effects of secondary structure and strand transfers on reverse transcription, at this rate it would take WDSV RT approximately 24 h to reverse transcribe the WDSV genome.

The results presented in this study represent an initial biochemical characterization of WDSV RT. The fact that the enzyme is a monomer in solution like MuLV RT further supports the evolutionary relatedness of WDSV and the C-type mammalian viruses. Comparison of the specific activities of WDSV and HIV-1 showed that the former is much lower. This difference could reflect suboptimal assay conditions, an inherently slower polymerization rate of WDSV RT, or it could merely indicate that the population of purified RT included inactive molecules. An active site titration of purified enzyme with inhibitor could address this question, but insufficient quantities of purified RT unfortunately prevented further testing. The thermosensitivity of WDSV could be explained by the lack of evolutionary pressure for function above about 10 °C, since infection takes place during the spawning season when the lake water is cold. Interestingly, another fish RT was reported long ago to exhibit a low temperature optimum. RT activity associated with C-type virus particles in lymphosarcoma tissue from Northern pike, a fish that inhabits lakes similar to those inhabited by WDSV, was partially purified and tested for activity at temperatures ranging from 5 to 40 °C (Papas et al., 1976, 1977). That enzyme was optimally active at 20 °C. Although the published studies did not directly distinguish heat inactivation from catalytic efficiency, the results can be interpreted to mean that the pike retrovirus RT was inactivated by temperatures above 20 °C, similar to WDSV RT. Analysis of RTs of other retroviruses that replicate in the cold, like WEHV-1 and -2, will be needed to establish the generality of RT thermosensitivity. A priori, it seems plausible that for an RT that works naturally in a cold environment, the catalytic activity might be adapted to be more efficient at low temperatures. However, the observation that the ratios of activity at 4 and 15 °C were similar for WDSV and HIV-1 contradicts this notion. Nevertheless, the data do not exclude the possibility that WDSV RT might have an especially high processivity and ability to synthesize through secondary structure at low temperature, compared with mammalian RTs. Further studies using natural templates with secondary structure are needed to address this possibility.

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References


