HIV-1 reverse transcriptase/pseudoknot RNA aptamer interaction has a binding affinity in the low picomolar range coupled with high specificity

Oliver Kensch§, Bernard A. Connolly#, Heinz-Jürgen Steinhoff§, Alistair McGregor#, Roger S. Goody§ and Tobias Restle§¶

§Max-Planck-Institut für molekulare Physiologie, Abteilung Physikalische Biochemie, Otto-Hahn-Straße 11, 44227 Dortmund, Germany.

#School of Biochemistry and Genetics, University of Newcastle, Newcastle upon Tyne, NE2 4HH, UK.

¶To whom correspondence should be addressed: Max-Planck-Institut für molekulare Physiologie, Abteilung Physikalische Biochemie, Otto-Hahn-Straße 11, 44227 Dortmund, Germany, Tel. +49 231 133 2364; Fax +49 231 133 2398, email: tobias.restle@mpi-dortmund.mpg.de

Running title: HIV-1 RT/pseudoknot RNA interaction
**Summary**

SELEX is a powerful method for the identification of small oligonucleotides which bind with high affinity and specificity to target proteins. Such DNAs/RNAs are a new class of potential chemotherapeutics which could block the enzymatic activity of pathologically relevant proteins. We have conducted a detailed biochemical study of the interaction of HIV-1 reverse transcriptase (RT) with a SELEX derived pseudoknot RNA aptamer. Electron paramagnetic resonance spectroscopy of site-directed spin-labeled RT mutants revealed that this aptamer was selected for binding to the "closed" conformation of the enzyme. Kinetic analysis showed that the RNA inhibitor bound to HIV RT in a two step process, with similar association rates to those described for model DNA/DNA and DNA/RNA substrates. However, the dissociation of the pseudoknot RNA from RT was dramatically slower than observed for model substrates. Equilibrium binding studies revealed an extraordinarily low $K_d$, of about 25 pM, for the enzyme/aptamer interaction, presumably a consequence of the slow off-rates. Additionally, this pseudoknot aptamer is highly specific for HIV-1 RT, with the closely related HIV-2 enzyme showing a binding affinity close to 4 orders of magnitude lower.
**Introduction**

HIV\(^1\) reverse transcriptase (RT), a key enzyme of the retroviral life cycle, catalyses the conversion of the single stranded genomic viral RNA into double stranded proviral DNA, which in turn is integrated into the host genome. The enzyme consists of an asymmetric heterodimer of two subunits, p66 and p51, possessing RNA- and DNA-dependent DNA polymerase and RNase H activities. The small subunit, p51, is derived from p66 by proteolytic cleavage of the C-terminal domain. Several X-ray structures of RT have been determined showing structural changes of the enzyme depending on whether the protein is bound to inhibitors, substrates or unliganded (for a recent review see 1). The overall fold of the p66 subunit has been compared with a right hand, consisting of subdomains termed fingers, palm, thumb, connection and RNase H.

RT is one of the main targets in the fight against AIDS. Drugs currently in use include nucleoside inhibitors such as AZT, 3TC, ddI, ddC, d4T, as well as non nucleoside inhibitors such as nevirapine, delavirdine and efavirenz, all of which target RT (for a review see 2). However, severe side effects and the rapid emergence of drug resistant mutants compromise the efficacy of these drugs. Recently, significant progress has been achieved by applying multi-drug combination therapy (for a review see 3). Here, at least two drugs against RT and one against the retroviral protease are taken simultaneously. Nevertheless, the underlying problems of this kind of chemotherapeutic treatment remain the same, urgently calling for alternative strategies to deal with viral infections such as AIDS.

Many different strategies have been discussed without, so far, leading to a genuine alternative to the currently used anti-AIDS drugs. However, one promising alternative approach could be the application of nucleic acid aptamers derived using *in vitro* selection. Aptamers bind with high affinity as well as specificity to their target proteins, often eliminating enzymatic activity.
Tuerk and Gold (5) have used SELEX to identify RNA aptamers against the HIV-1 RT. The analysis of the isolated aptamers revealed a consensus sequence that resulted in the formation of an RNA pseudoknot (6). The interactions between SELEX RNA pseudoknots and HIV-1 RT have been analyzed in some detail using biochemical studies and chemical modification (7, 8).

Pseudoknots are defined as loop regions base pairing with complementary sequences, outside the loop, in the same RNA molecule (9, 10). The pseudoknot fold is a widespread structural motif found in all kinds of RNA, including coding and noncoding regions of cellular mRNAs, viral RNAs, ribosomal RNAs, and snRNAs. Although not all of the functions of this RNA secondary structure motif are fully understood, pseudoknots play an important role in ribosomal frameshifting, in transcriptional read-through, as internal ribosomal entry sites, as translational enhancers, and are key components of ribozymes (11) and cis-acting elements important for the initiation of viral replication (for a review see 12). Several NMR studies and one high resolution X-ray study of pseudoknots have been reported (13-19).

The X-ray structure of HIV-1 RT complexed with an RNA pseudoknot inhibitor has been solved recently (see Figure 1; 20). This is the first example of an X-ray structure of such an pseudoknot aptamer bound to its target protein. The RNA ligand binding surface lies within the nucleic acid cleft of the enzyme, between the polymerase and RNaseH active sites, and partially overlapping the binding surface of duplex DNA substrates. The pseudoknot is kinked by 60° from the co-axial stacking of stems 1 and 2, thus optimizing the extensive contacts between the RNA inhibitor and both subunits of the heterodimeric enzyme. The protein/RNA interaction stabilizes the "closed" conformation of the enzyme, where the fingers and thumb domains of the large subunit are in close contact. Further, we suggested that the SELEX procedure appears to have identified an RNA molecule whose uncomplexed solution structure
is very similar to its structure when bound to RT and which binds to the unliganded conformation of RT.

Here we present the first detailed biochemical study of the this enzyme/inhibitor interaction. Using electron paramagnetic resonance (EPR) spectroscopy of site-directed spin-labeled RT mutants, we show that the relative positions of the fingers and thumb domains of the large subunit are virtually indistinguishable whether or not RNA is bound. This proves, for the first time, that RT in solution can adopt the closed conformation seen in the X-ray structure by Rodgers et al. (21) and might be an important feature of selecting for tight binding ligands. An analysis of the binding equilibrium of the protein/inhibitor complex revealed astonishingly tight binding, with a $K_d$ in the low picomolar range, about 2-3 orders of magnitude lower than the $K_d$ of about 5 nM reported earlier by Tuerk et al. (6). To our knowledge, this is the first report of a SELEX derived RNA ligand showing such a tight binding for a known nucleic acid binding target protein.
**Experimental procedures**

**Mutagenesis of reverse transcriptase**

To label RT with either spin labels or fluorophors in a site directed manner, the naturally occurring cysteines had to be replaced by serines and cysteines had to be introduced at the desired positions. In a first step, the mutant RT $p66^{C38S,C280S}/p51^{C280S}$ was generated. The cysteine at position 38 in the small subunit is not solvent accessible and consequently does not interfere with site-directed labeling (Kensch, unpublished data). In a second step cysteines at certain positions were introduced as given in the text.

Mutant RT was prepared by site directed mutagenesis using PCR (22). The mutations were introduced into the plasmids pRT166 (23) and p6HRT51 (24). The generated plasmids were transformed into *Escherichia coli* (E. coli) M15/pDMI.1 (25) resulting in expression systems for mutated p66 and mutated his-tagged p51.

**Protein Purification**

Recombinant heterodimeric wild type HIV-1, HIV-2 and EIAV RT were expressed in *E. coli* and purified as described before (23, 26, 27). Enzyme concentrations were routinely determined using an extinction coefficient at 280 nm of 260450 M$^{-1}$cm$^{-1}$ (HIV-1 RT), 238150 M$^{-1}$cm$^{-1}$ (HIV-2 RT) and 223180 M$^{-1}$cm$^{-1}$ (EIAV RT). The purified RTs were free of nuclease contamination.

Mutant RTs were purified according to a protocol described previously (28). Co-homogenization of *E. coli* cells expressing p66 or p51, respectively, led to reconstruction of heterodimeric p66/p51 RT. Analysis of the mutant proteins by a standard RT assay (see below) showed indistinguishable polymerase activity as compared to the wild type enzyme.
**Labeling of RT mutants**

Spin labeling of the introduced cysteine residues at position 24 and 287 in p66 was achieved by the following procedure. In order to reduce any disulfide bonds, 2 µl 1 M DTT was added to 200 µl of a 44 µM solution of mutant RT (p66\(^{\text{W24C, C38S, C280S, K287C}}\) /p51\(^{\text{C280S}}\)) in buffer A. After 30 min at 4° C a 2-fold excess of 18/36mer DNA/DNA p/t was added to the solution. The resulting RT-p/t complex was separated from excess DTT using a Sephadex G25 gel filtration column (Pharmacia). The eluted complex was collected in a tube containing 2 µl of 100 µM (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methane-thiosulfonate (MTSSL; Toronto Research Chemicals, Inc.) in DMSO. After 16 h at 4° C, excess spin label and bound p/t were removed by purifying the enzyme over a Ni\(^{2+}\)-nitrilotriacetic acid-Sepharose column (Qiagen). The bound protein was washed extensively with a buffer containing 1 M NaCl and eluted with 0.3 M imidazole. Subsequently the protein solution was concentrated and buffer was changed (50 mM Tris-HCl, pH 8.0, 25 mM NaCl, 6 mM MgCl\(_2\), 10% glycerol) using centrifugal filters (Millipore). Finally samples were shock frozen in liquid nitrogen and stored at –80° C. The labeled cysteine/RT ratio estimated from double integration of the EPR spectra and absorption measurements of protein (280 nm) was found to be > 90%. Analysis of the spin labeled protein by a standard RT assay (see below) show polymerase activity which was indistinguishable from that of wild type enzyme.

The labeling of the HIV-1 RT mutant (p66\(^{\text{C38S, C280S}}\)/p51\(^{\text{C280S, K281C}}\)) used for the FRET experiments at position p51\(^{281C}\) with the fluorophor Alexa488 was performed according to the instructions given by the manufacturer (Molecular Probes). Equilibrium as well as kinetic measurements of DNA/DNA p/t binding to this fluorescently labeled RT gave similar values to those obtained for the wild type protein (data not shown).
RNA Preparation

The 33 nt pseudoknot RNA (sequence: 5'-GGGAGAUUUCGUUUUCAGUGGGAAAAACUGAA) was prepared in a standard 10 ml T7 reaction mixture for in vitro transcription and purified by gel electrophoresis as described previously (20, 29). The RNA was refolded at a concentration of 200 - 300 µM at 65° C for 5 min followed by slow cooling to room temperature in 20 mM cacodylate buffer pH 6.5, 25 mM NaCl and 5 mM MgCl₂. 5'-end-labelling of the RNA with T4 polynucleotide kinase (New England Biolabs) was performed as described previously (30). Dephosphorylation of the in vitro transcribed RNA prior to endlabeling was carried out according to standard procedures (31). The fluorescent-labeled 5'-HEX-(hexachlorofluorescein)-pseudoknot RNA was synthesized and HPLC-purified as previously described (32). HEX phosphoramidite was obtained from Glen Research (Sterling, VI, USA). This chemically synthesized RNA consists of 28 residues missing the last 5 nucleotides at the 5'-end. Final purity was > 97 % as assessed by HPLC.

Buffer

Protein/RNA interactions were routinely analyzed at 25° C in a buffer containing 50 mM Tris/HCl pH 8.0, 50 mM KCl, 1 mM DTT and 10 mM MgCl₂ (standard buffer). Additionally, some of the experiments were also performed in a buffer containing 200 mM KOAc, 50 mM Tris/HCl pH 7.7 and 10 mM DTT (6). EPR measurements were performed in a buffer containing 50 mM Tris-HCl pH 7.0, 12 mM NaCl and 5 % glycerol.

Polymerase Activity Determination

RNA-dependent DNA polymerase activity on poly(rA)/oligo(dT)₁₂₋₁₈ substrates was measured by a standard assay described previously (33, 34) with 2.8 nM of RT for 10 min at 37° C in a
buffer containing 50 mM Tris/HCl pH 8.0, 80 mM KCl, 5 mM DTT, 6 mM MgCl₂ and 0.05 % (v/v) Triton X-100.

**Filter Binding Assay**

Protein and 5′-32P-labeled RNA were mixed in standard buffer and incubated at 25° C for 10 min. An aliquot of this mixture was filtered under suction through a prewet (standard buffer) nitro-cellulose filter (Schleicher & Schuell BA85) and rinsed with 4 ml of standard buffer. Radioactivity retained on the filters was measured by scintillation counting.

**Fluorescence Equilibrium Measurements of RT/RNA Binding**

The affinity of the different RTs for the pseudoknot RNA was measured both by displacing a fluorescently labeled 18/36-mer DNA/DNA p/t bound to RT and by titrating increasing amounts of RT with 5′-HEX-labeled RNA.

The fluorescently labeled DNA-primer was synthesized by coupling the phosphoroamidite dye 6-FAM, a fluorescein derivative, directly to the 5′-end of the oligodeoxynucleotide during DNA synthesis according to the recommendation of the manufacturer (Applied Biosystems). The titrations were performed using an SLM AB2 spectrofluorometer. To monitor the fluorescence change upon displacing the labeled p/t from RT, the samples were excited at 492 nm and the emission intensity was measured at 516 nm. These competitive titrations were evaluated using the program Scientist (MicroMath), which allows the user to define the system under investigation as a series of parallel equations defining (in this case) each discrete equilibrium, the relationship between the total and free concentrations of the components, and the way in which the observable signal is generated. The $K_d$ of the 18/36mer DNA/DNA p/t was determined independently (30, 35) and kept constant during the fit procedure (primer
sequence: 5′-TCCCTGTTCGGGCGCCAC-3′; template sequence 5′-
TGTGGAAAATCTCATGCAGTGGCGCCCGAACAGGGA-3′).

To monitor the fluorescence change upon binding of RT to the 5′-HEX-labeled pseudoknot RNA, the samples were excited at 538 nm and the emission intensity was measured at 556 nm. Data were fitted to a quadratic equation analogous to the one given by Müller et al. (36) using the program Grafit (Erithacus Software). Values for the dissociation constant (K_d), the amplitude of the fluorescence change and the RNA concentration were allowed to vary during the fit procedure.

**Rapid kinetics of RT/RNA interaction**

Experiments on the kinetics of the association of HIV-1 RT with the pseudoknot RNA were performed using a stopped flow apparatus (High Tech Scientific, Salisbury, England). 25 nM of a Alexa488-labeled HIV-1 RT (final concentration) was rapidly mixed with increasing 5′-HEX-labeled pseudoknot RNA concentrations (50 - 300 nM). Collection and analysis of the data was done as described previously (27, 30). Excitation of the Alexa488-labeled protein was at 435 nm and detection of the donor quenching due to fluorescence resonance energy transfer to the HEX-fluorophor was through a bandpass filter (520 nm). Data were fitted using a double exponential equation. The rate of the first phase (k+1) is dependent on the concentration of RT and corresponds to the formation of the collision complex. The rate k+2 of the second phase is largely concentration independent and arises from a conformational change of the RT-RNA complex after formation of the collision complex.

**EPR Measurements**

Continuous wave EPR experiments were performed using home made X-band EPR spectrometers equipped with a dielectric resonator (Bruker) or with an H103 cavity (AEG). The
magnetic field was measured with a B-NM 12 B-field meter (Bruker). Spin labeled RT samples were loaded into EPR quartz capillaries (50 µL for low temperature measurements, otherwise 5 µL) at a final RT concentration of 100 - 200 µM. Spectra were recorded with a modulation amplitude of 1.5 G and the microwave power adjusted to between 0.2 mW and 0.6 mW. A modified Oxford ESR 9 variable temperature accessory allowed stabilization of the sample temperature between 80 K and 330 K. The whole apparatus was controlled by a personal computer, which also performed 12 bit analog-to-digital data acquisition. EPR powder spectra simulations were performed according to the method described by Steinhoff et al. (37).

The spin-spin interaction between two spin labels attached to a protein is composed of static dipolar interaction, modulation of the dipolar interaction by the residual motion of the spin label side chains and exchange interaction. For temperatures below 200 K the residual motion of the nitroxide side chain is strongly restricted and the static dipolar interaction leads to considerable broadening of the spectrum if the inter-spin distance does not exceed 25 Å. A detailed line shape analysis allows determination of absolute values of the inter-spin distance in the range from about 10 Å to 25 Å (37-40). For certain cases the modulation of the dipolar interaction due to the motion of the spin labels allows an estimation of the inter-residue distances at room temperature (41). Exchange interaction was found to contribute significantly to the line broadening for distances of less than 10 Å, since partial overlap of the nitrogen pi-orbitals of the two interacting nitroxides is required (42, 43).
Results

Affinity of HIV-1 RT for the pseudoknot RNA by equilibrium binding

Binding measurements to determine the $K_d$ of the RT/RNA complex were performed using three different approaches. Figure 2A shows a displacement titration using a fluorescently labeled DNA/DNA p/t, for which the affinity has previously been determined (35). When this p/t binds to RT its fluorescence is quenched. A complex was formed between RT and the p/t and increasing amounts of pseudoknot RNA added. The fluorescence increase observed upon displacement of the fluorescent p/t into solution was measured, as shown in Figure 2A. A $K_d$ of 25 pM was calculated from a least squares fit of the data to a model describing both equilibria (see experimental procedures).

Figure 2B shows titration of increasing amounts of HIV-1 RT with a 5'-HEX-(hexachlorofluorescein)-labeled pseudoknot RNA. Data were fitted to a quadratic equation yielding a $K_d$ of 24 pM. These results indicate that the chemically synthesized RNA, labeled with a fluorophor on the 5'-end (see experimental procedures), shows a similar affinity for RT to the *in vitro* transcribed RNA used in figure 2A.

However, in both figures 2A and 2B the concentration of the substrates is 2-3 orders of magnitude higher than the determined $K_d$ value and therefore the $K_d$s derived show a rather large error, being in the same range as the fitted value. Due to the limiting fluorescence signal of the probes it was not possible to reduce the substrate concentrations below 1 nM. To overcome the drawbacks of fluorescence based measurements radioactive labeling was used, enabling the use of substrate concentrations in the pM range. Figure 2C shows a filter binding assay where 5'-$^{32}$P-labeled pseudoknot RNA was titrated with increasing amounts of RT. However, upon dilution of the RNA from the stock solution (which had a concentration in the µM range) to the working solution in the low pM range, about 90 % of the RNA was lost,
probably due to molecules irreversibly absorbing to the Eppendorf tube. Several attempts to minimize this problem were only partially successful. However, as the RNA is highly labeled it was possible to correct for these losses. Fitting of the experimental data, shown in figure 2C, to a quadratic equation yielded a $K_d$ of about 40 pM, in good agreement with the fluorescence-based approaches. However, this $K_d$ is dependent on the assumption that dilute solutions of RT do not result in absorption to the Eppendorf tube, as seen with the RNA. If such absorption (which cannot easily be corrected for) takes place the effective protein concentration would be reduced, leading to a lower $K_d$ value than determined. Therefore the figure of 40 pM should be regarded as an upper estimate.

Measurements were performed routinely in standard buffer (50 mM Tris/HCl pH 8, 50 mM KCl, 1 mM DTT and 10 mM MgCl$_2$) with refolded pseudoknot RNA. Performing the experiments in the buffer used to select the pseudoknot (50 mM Tris/HCl pH 7.7, 200 mM KOAc and 10 mM DTT) and carry out previous experiments (6), with RNA which was not folded, gave similar results.

**Kinetics of RT/RNA association**

Stopped flow experiments were performed to analyze the association of RT with RNA. Binding of RT to the 5'-HEX-labeled pseudoknot RNA results in quenching of the fluorescence signal of about 6% (see Figure 2B). This rather small signal change was, however, not sufficient to obtain reasonable data in stopped flow experiments. We therefore used FRET (fluorescence resonance energy transfer) to enhance the signal. The Alexa$_{488}$-florophor (absorption and emission maxima: 493 and 520 nm, respectively) covalently linked to a cysteine at position 281 in the small subunit of the heterodimeric enzyme was used as donor and the HEX-fluorophor (absorption and emission maxima: 538 and 555 nm, respectively) on the 5'-end of the RNA as acceptor. From the X-ray structure of the
protein/RNA complex it could be predicted that the two fluorophors are about 20 Å apart (see Figure 1). Upon binding of the RNA to the protein a quenching of the donor fluorescence of about 20 % could be observed. Independent measurements demonstrated that the Alexa488 labeled enzyme had comparable behaviour to wild type RT with respect to enzymatic activities, substrate binding and affinity for the pseudoknot (data not shown).

Figure 3A shows a typical stopped flow experiment. The data obtained could be fitted best to an equation with two exponential terms. The fast first phase was dependent on the RT concentration, whereas the slower one was not. In Figure 3B the dependence of the first phase of pseudoknot binding (observed pseudo-first-order rate constant) on RT concentration is shown. The rate constants $k_{+1}$ and $k_{-1}$ are given by the slope of the line and the intercept with the y-axis, respectively (44). For the first step in pseudoknot binding values for $k_{+1}$ and $k_{-1}$ of $5.6 \times 10^8$ M$^{-1}$ s$^{-1}$ and 10 s$^{-1}$ were obtained, in the same range as those observed previously for DNA/DNA and DNA/RNA binding (45).

**Analysis of the RT/RNA dissociation**

Attempts to measure the dissociation rate of the protein/RNA complex using stopped flow did not result in an appreciable signal change over the maximal useful time range of the instrument, indicating that this process might be too slow to be measured by this approach. Thus, the filter binding assay was applied to determine the dissociation rate of the complex. 25 nM of RT was complexed with 20 nM of $^{32}$P-labeled RNA and then mixed with 2 µM of unlabeled pseudoknot. Analyses of the amount of radioactive pseudoknot that remained bound at different times (Figure 4) gave data that fitted to a single exponential, yielding an observed dissociation rate of 0.0002 s$^{-1}$. Essentially the same rate constant was determined using the 5'-HEX-labeled pseudoknot in a fluorescence spectrofluorimeter (data not shown).
Binding of RT to the pseudoknot RNA by kinetic measurements

The results outlined above, show that the binding of the pseudoknot RNA to HIV-1 RT can be best described as a two step process. Scheme 1 summarizes the results obtained form the different kinetic experiments. RT and pseudoknot RNA initially form a collision complex with a second order rate constant of $5 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ followed by a slower concentration independent isomerization with a rate constant of about $5 \text{ s}^{-1}$. The rate constants determined for the reverse reaction are $0.0002 \text{ s}^{-1}$ and $10 \text{ s}^{-1}$, respectively. The latter was indirectly derived from the y-axis intercept of a linear fit of RNA concentration versus observed pseudo-first-order rate and therefore is subject to a rather large error (see Figure 3B). Efforts to observe $k_1$ directly by performing double mixing stopped flow experiments did not yield interpretable data (data not shown). A $K_d$ of about 0.8 pM can be calculated from these rate constants, about 30 times lower than determined using equilibrium measurements.

Affinity of HIV-2 and EIAV RT for the pseudoknot

To investigate the specificity of the interaction of the pseudoknot with HIV-1 RT binding studies were carried out with the closely related RTs of HIV-2 and EIAV (equine infectious anemia virus). Figure 5 shows the effect of adding increasing amounts of these RTs to the 5'-HEX-labeled pseudoknot. Fitting the experimental data to a quadratic equation yielded $K_d$s of 85 nM and 118 nM for the HIV-2 and EIAV complexes, respectively. Interestingly the signal change upon binding of these RTs to the fluorescently labeled RNA is much larger than for HIV-1 RT. In both cases the fluorescence signal was quenched by about 50 % compared to about 6 % in the case of HIV-1 RT. To ensure that the fluorophor is not interfering with binding to the HIV-2 and EIAV RT, we analyzed the interaction by performing displacement titrations using a fluorescently labeled DNA/DNA p/t as described above. These experiments gave essentially the same $K_d$ values (data not shown).
Effect of the RNA on polymerase activity of different RTs

To further illustrate the exceptional specificity of the pseudoknot RNA for the HIV-1 enzyme we performed standard RT assays using poly(rA)/oligo(dT) as substrate. The reactions were started with preincubated RT/RNA complexes. Figure 6 shows the effect of the pseudoknot RNA on the polymerase activity of HIV-1, HIV-2 and EIAV RT. At pseudoknot concentrations where the HIV-1 enzyme is fully inhibited with respect to the polymerase activity there is only a minor effect on HIV-2 and EIAV RT, as would be predicted from the different $K_d$ values evaluated above. The difference in the observed $K_i$ values (defined as the pseudoknot concentration that inhibited polymerase activity by 50%) is given by a factor of about $3.5 \times 10^4$.

EPR measurements

Site directed spin labeling has emerged as a powerful technique for exploring the structure and dynamics of both soluble and membrane proteins (for a recent review see 46). We have applied this method to determine in solution the relative positions of the fingers and thumb domains of the p66 subunit of RT, both as an unliganded apo-enzyme and in the presence of bound nucleic acids, and have compared these data with data obtained from X-ray analysis (for a review see 1). RT was mutagenised to introduce two unique cysteines in the p66 subunit. Subsequent reaction with a thiol specific nitroxide resulted in an RT variant carrying two spin labels, one at the tip of the fingers and the second in the thumb domain (amino acids 24 and 287 respectively, see Figure 1). EPR spectroscopy was used to determine the distance between these spin labels.

The EPR spectra of spin-labeled RT liganded to either a DNA/DNA p/t (18/36-mer) or the pseudoknot RNA were measured at 170 K in frozen solution to exclude dynamic effects and motional averaging of the dipolar broadening. Room temperature measurements ($T = 293$ K)
were performed to characterize the mobility of the spin label side chains and to study spin-spin interaction at a more physiological temperature. The EPR spectra were normalized to represent the same number of spins. The results, shown in Figure 7, reveal considerable line broadening for the RT bound to RNA compared to the enzyme bound to DNA. Powder spectra simulations were performed to determine absolute values for the inter-spin distances (37). The experimental spectra are generally composed of species with different relative nitroxide orientations and inter-spin distances because of the flexibility of the spin label side chains and the variety of conformational substrates of the proteins in frozen solution. A fitting of simulated EPR spectra to the experimental data with the assumption of a Gaussian inter-spin distance distribution yields an average inter-spin distance, \( d \), and the distance distribution width, \( \sigma \), which also accounts for small amounts of singly spin labeled proteins. The parameters which describe the EPR spectrum in the absence of any spin-spin interaction were fixed according to the values obtained from the fitting of simulated powder spectra to the experimental data of spin labeled proteins in a similar environment (47). The respective values are given in the legend of Figure 7. The experimental and simulated spectra with inter-spin distances > 21 Å for the DNA bound species and 13 Å for the RNA bound structure agree well (Figure 7).

At room temperature the spin label side chain motion occurs in the ns time range, and the nitroxide mobility and the flexibility of the protein backbone are directly reflected in the EPR absorption line shape. The small apparent hyperfine splitting visible in the spectrum of the DNA-bound RT indicates high mobility of the nitroxide side chains. This spectral shape is typical for nitroxide side chains bound to flexible loop regions as revealed by the comparison with spectra of MTSSL bound to the E-F cytoplasmic loop of bacteriorhodopsin (48). The lack of any additional spectral component of considerable amplitude reveals that the dynamics of both nitroxide side chains must be very similar. In the RNA-bound species of RT, the
apparent hyperfine splitting is only slightly increased, indicating only minor changes in the
tertiary interaction of the nitroxides compared to the DNA bound structure. However, the line
width is substantially increased, which must be due to spin-spin interaction. The spectrum of
the RNA-bound RT can be fit reasonably well by the convolution of a Lorentzian function
with the spectrum of the DNA bound species. The non-broadened contribution to the
spectrum is most probably due to a small fraction of singly labeled species, which amounts to
less than 10%. The broadening appears to be homogeneous across the spectrum, and the line
width at half height, $\Delta H$, of the Lorentzian provides a quantitative measure of the spin-spin
interaction (41). The best fit is obtained with $\Delta H = 6$ G, which yields an inter-spin distance of
12.5 Å according to the empirical calibration given by Mchaourab et al. (41). Due to the high
flexibility of the nitroxides and since we do not distinguish between exchange and dipolar
interaction, this value has to be regarded as an estimate of the average inter-spin distance.
Additionally, as the line width depends on $r^{-6}$, the average is weighted in favour of molecules
with smaller inter-spin distances. With this in mind, the estimated distance value agrees well
with that determined from the low temperature experiment.

At room temperature, the spectrum of the substrate free RT is nearly indistinguishable from
that of the RNA bound species. Since the spectral shape of this mutant is determined by the
spin-spin interaction, this result is strong evidence that the finger-thumb distance of the main
fraction of the free RT is identical to that of the RNA bound structure.
Discussion

Comparing the X-ray structure of the pseudoknot RNA / RT complex (20) with that of RT bound to a DNA/DNA p/t (49, 50), reveals that the RNA makes more extensive contacts with the enzyme than the natural substrate does. The DNA duplex contacts mainly the p66 subunit of the RT heterodimer, showing interactions close to the polymerase active site (fingers and thumb domain) and the RNaseH active site. The inhibitory RNA also shows interactions with these regions of the enzyme. Additionally, there are extensive interactions with the small, p51, subunit. The RNA, therefore, sits snugly in the nucleic acid binding cleft; in contrast to DNA/DNA duplexes, which appear to "float" above this cleft. For the complex of RT with a DNA duplex a $K_d$ of about 2 nM has previously been determined (35). On the basis of the available structural information which indicate less extensive interactions of DNA/DNA as compared to the RNA inhibitor, we performed a detailed biochemical examination of the RT/pseudoknot RNA interaction. In particular, we suspected the binding might be considerably stronger than what has been published by others (6).

Equilibrium binding experiments using fluorescently as well as radioactively labeled RNA yielded $K_d$ values of about 25 pM for the enzyme inhibitor complex. As described above (see results) binding studies in the low pM range are technically problematic due to experimental limitations which are difficult to overcome. We therefore regard the value of 25 pM as an upper limit, meaning that the interaction could be even tighter. Interestingly, we observed similar binding affinities regardless of the salt concentration of the buffer (50 - 200 mM). Additionally, refolding of the RNA in a buffer containing MgCl$_2$ using a standard protocol was not necessary to obtain tight binding. The only difference seen was the time required for the fluorescence signal to become stable in titration experiments when the RNA was not refolded, indicating that the proper folding might be induced and stabilized upon interacting
of the pseudoknot with RT. However, it is not known whether Mg$^{2+}$ is important for the folding of this RNA in the absence of the enzyme.

The kinetic analysis of the RT / pseudoknot interaction revealed that binding occurs in two steps, previously shown for DNA/DNA or DNA/RNA binding to the enzyme (45). The observed on-rates for complex formation are in the same range as for the nucleic acid duplex substrates, whereas the off-rates are much slower explaining the extraordinarily tight binding. Calculating the $K_d$ of the complex using the kinetic constants given in scheme 1, gives a value of 0.8 pM, about 30 times lower than what has been determined via the equilibrium binding experiments. This obvious discrepancy could be explained by either the $k_{-1}$ value not being entirely correct, since it was determined indirectly, or by the determined equilibrium $K_d$ being too high for technical reasons, as described above, or a combination of both. Nevertheless, the two $K_d$ values are in reasonable agreement when the limitations of the techniques used are considered.

An important aspect concerning the pseudoknot RNA aptamer is the specificity of the interaction with HIV-1 RT. This is not only interesting in terms of the selectivity index of this inhibitor, but also with respect to the potential of the SELEX method to select for highly specific ligands. Binding studies with HIV-2 and EIAV RTs yielded $K_d$s of 85 and 118 nM, respectively. This dramatic reduction in affinity in comparison to HIV-1 RT was an especially surprising finding since the HIV-2 RT used in this study shows 60 % identity and 13 % homology to the HIV-1 enzyme. On the basis of this result, we propose that in addition to the overall fold of the pseudoknot, there must be site-specific interactions between protein and RNA which are responsible for the tight binding to HIV-1 RT, since it is likely that the HIV-2 enzyme adopts a comparable structure and therefore should be capable of forming similar overall interactions. This could be an explanation for the sequence conservation seen in stem 1 among the minimal Tuerk-type pseudoknots (8). Unfortunately, the resolution of the X-ray
structure of the RT/RNA complex (about 4.8 Å) is not sufficient to clearly identify such interactions.

The conformation of RT observed in the X-ray structure of the RNA complex resembles the "closed" conformation, where fingers and thumb of p66 are in close contact, also seen in the structure of the unliganded enzyme (21, 51). This conformation is stabilized by the RNA through extensive electrostatic interactions with several basic residues in helix I of the p66 thumb and in the p66 fingers domain. From the crystal-structure of Rodgers et al. (21), it cannot be deduced whether RT adopts this closed conformation in solution since it cannot be ruled out that this structure is induced and/or stabilized by crystal packing. Consequently, there is no clear answer to the question of whether the pseudoknot RNA was selected against the closed conformation of RT, as recently proposed (20) or, alternatively, that the protein/RNA interaction has induced a closure of the thumb domain relative to the fingers domain. To address these questions, we performed EPR measurements, analyzing the inter-spin distances of spin-labels attached to the tip of the fingers and thumb domain in p66. The solution structure at 293 K of RT complexed with the RNA pseudoknot shows a distance of 13 Å between the spin labels at positions Cys24 and Cys287 in the large subunit. This value is in excellent agreement with the distance of 12 Å between Cβ from Trp24 and Lys287 found in the crystal structure of the RT/pseudoknot-complex (20). Furthermore, the EPR spectra of unliganded and pseudoknot-complexed RT are virtually indistinguishable, clearly showing for the first time that RT adopts the closed conformation in solution at room temperature. Thus, the pseudoknot RNA appears to have been selected against this structure, indicating that neither the RNA nor the protein undergoes significant conformational change upon complex formation, which would further account for the extraordinarily low Kd for this interaction. Typically, RNA aptamers selected for tight binding to protein targets show binding constants in the low nM - µM range (52). Occasionally tighter binding with Kds of 100 - 500 pM is
reported. However, to our knowledge an RNA aptamer with a binding constant of $\leq 25$ pM has not been described so far. This aptamer shows at least a 100 fold tighter binding than the natural substrate, exceptional specificity and a broad working range concerning buffer conditions, making it an encouraging drug candidate for the treatment of HIV infections. Experiments are in progress to evaluate the inhibitory potential of this pseudoknot RNA in cell tissue culture.
Acknowledgements

We thank Karin Vogel-Bachmayr and Martina Wischnewski for excellent technical assistance, Manfred Souquet for purifying the EIAV RT, Paul Rothwell for purifying the Alexa488-labeled HIV-1 RT, Joachim Jäger for providing Figure 1, and Jochen Reinstein and Birgitta Wöhrl for fruitful discussions. BAC was supported by a UK-MRC Senior Research Leave Fellowship. Part of this work was carried out while BAC was on sabbatical in Dortmund. A CIBA Fellowship Trust award contributed towards the cost of this sabbatical.

1 The abbreviations used are: HIV, human immunodeficiency virus; EIAV, equine infectious anemia virus; RT, reverse transcriptase; SELEX, Systematic Evolution of Ligands by EXponential Enrichment; EPR, electron paramagnetic resonance; FRET, fluorescence resonance energy transfer; p/t, primer/template; HEX, hexachlorofluorescein
References


**Figure Legends**

**Fig. 1.** Structure of the HIV-1 RT pseudoknot RNA complex. The p66 subunit is shown in blue, the p51 subunit in white and the RNA in red. Surface residues < 5 Å away from the RNA aptamer are coloured in orange. The positions of the two spin labels used for the EPR measurements at the tip of the fingers and thumb domain of the p66 subunit are indicated.

**Fig. 2.** Equilibrium titrations of HIV-1 RT and pseudoknot RNA. (A) Displacement titration of fluorescent p/t bound to HIV-1 RT with pseudoknot RNA. A complex of FAM-labeled 18/36-mer DNA/DNA (25 nM) and RT (40 nM) was titrated with increasing amounts of competitor. The curve shows the best fit by least-square fitting to the model describing the two binding equilibria from which a dissociation constant of 25 pM (± 17) for the pseudoknot was obtained (see experimental procedures). The sequence of the DNA/DNA p/t is given under experimental procedures. (B) Titration of 5′-HEX-labeled pseudoknot RNA (3 nM) with increasing amounts of HIV-1 RT. The curve shows the best fit to a quadratic equation describing the binding of the RNA to a single site in the heterodimeric enzyme. The fit gives a value of 24 pM (± 39) for the $K_d$. (C) Filter binding assay of radiolabeled RNA with RT. 8 pM of 5′-32P-labeled pseudoknot RNA was titrated with increasing amounts of RT. The curve shows the best fit to a quadratic equation yielding a $K_d$ of 40 pM (± 6).

**Fig. 3.** Kinetics of the binding of 5′-HEX-labeled pseudoknot RNA to a Alexa488-labeled HIV-1 RT (amino acid 281 in p51). (A) A typical stopped flow result is shown. A 25 nM sample of RT was rapidly mixed with 250 nM of RNA. Excitation was at 435 nm and the donor emission was detected via a bandpass filter (520 nm). Fitting of the experimental data to a double exponential equation gave rates of 151 (± 6) s$^{-1}$ and 4.9 (± 0.4) s$^{-1}$, respectively. (B)
Dependence of the pseudo-first-order rate constant of pseudoknot binding on the RNA concentration. A constant concentration of fluorescently labeled RT (25 nM) was mixed with increasing amounts of 5'-HEX-labeled pseudoknot. \( k_{+1} \) and \( k_{-1} \) were determined by the slope of the linear fit and the intercept of the line with the y-axis and yielded values of \( 5.6 \times 10^8 \pm 0.4 \) M\(^{-1}\) s\(^{-1}\) and \( 10 \pm 8 \) s\(^{-1}\), respectively.

**Fig. 4.** Analysis of the dissociation of the protein/RNA complex using a filter binding assay. 25 nM of HIV-1 RT was preincubated with 20 nM 5'-\( ^{32} \)P-labeled pseudoknot RNA and then mixed with 2 \( \mu \)M of unlabeled pseudoknot RNA. Aliquots were analyzed at different time points by spotting onto a nitro-cellulose filter, rinsing with 4 ml of binding buffer and scintillation counting. The curve shows the best fit to a single exponential equation yielding an off-rate of 0.0002 s\(^{-1}\).

**Fig. 5.** Binding of the pseudoknot RNA to HIV-2 and EIAV RT. 5'-HEX-labeled pseudoknot RNA (7 nM) was titrated with increasing amounts of HIV-2 (A) and EIAV (B) RT, respectively. The curves show the best fit to a quadratic equation yielding \( K_d \)s of 85 (± 4) nM and 118 (± 4) nM for the HIV-2 and EIAV enzyme, respectively.

**Fig. 6.** Standard RT assay with different RTs in the presence of pseudoknot RNA. RNA-dependent DNA polymerase activity on poly(rA)/oligo(dT)\(_{12-18}\) substrates (ca. 1.2 \( \mu \)M with respect to the free 3'-ends) was measured for 10 min at 37° C. 2.8 nM of HIV-1 (circles), HIV-2 (squares) and EIAV (rhombi) RT were preincubated with increasing amounts of pseudoknot RNA. The reaction was initiated by the addition of the RT/RNA complexes to the assay mixture.
Fig. 7. EPR spectra of the spin labeled RT double mutant p66$^{W24C_R1,K287C_R1}$ (R1 = MTSSL; see experimental procedures). (A) Spectra of RT + DNA/DNA (grey trace) and RT + pseudoknot RNA were recorded at 170 K and normalized to constant spin number. The dotted traces show fits of dipolar broadened powder spectra with inter-spin distances of $> 21$ Å (RT + DNA/DNA) and 13 Å (RT + pseudoknot RNA). Parameters taken from spectra of non-interacting spin labels and kept fixed during the fitting procedure were: $g_{xx} = 2.0089$, $g_{yy} = 2.0065$, $g_{zz} = 2.0026$, $A_{xx} = 5.4$ G, $A_{yy} = 4.9$ G, $A_{zz} = 36.5$ G and a field independent line shape function composed of a superposition of 53% Lorentzian and 47% Gaussian of 4.8 G and 4.1 G width, respectively. (B) Room-temperature spectra of the RT double mutant with bound DNA/DNA or pseudoknot RNA (heavy traces) and without substrate (light trace) normalized to constant spin number. The dashed trace shows the convolution of the of the spectrum of the DNA/DNA bound RT with a Lorentzian broadening function of line width, $\Delta H = 6$ G, that gives the best fit to the dipolar broadened spectral fraction of the pseudoknot RNA bound RT.
Figure 1 (Kensch et al.)
Figure 2 (Kensch et al.)
Figure 3 (Kensch et al.)
Figure 4 (Kensch et al.)
Figure 5 (Kensch et al.)
Figure 6 (Kensch et al.)
Figure 7 (Kensch et al.)
\[ 5 \times 10^8 \text{ M}^{-1}\text{s}^{-1} \quad \text{ca. 5 s}^{-1} \]

\[
\text{RT + RNA} \quad \leftrightarrow \quad \text{RT-RNA} \quad \leftrightarrow \quad \text{RT*RNA}
\]

ca. 10 s\(^{-1}\) \quad \quad \quad 0.0002 \text{ s}^{-1}

* This sign distinguishes structural states of the system that otherwise have the same composition

**Scheme 1 (Kensch et al.)**
HIV-1 reverse transcriptase/pseudoknot RNA aptamer interaction has a binding affinity in the low picomolar range coupled with high specificity

Oliver Kensch, Bernard A. Connolly, Heinz-Jürgen Steinhoff, Alistair McGregor, Roger S. Goody and Tobias Restle

J. Biol. Chem. published online April 4, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M001309200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2000/04/04/jbc.M001309200.citation.full.html#ref-list-1