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Reverse Transcriptase in Motion: Conformational Dynamics of Enzyme-Substrate Interactions

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Abstract

Human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) catalyzes synthesis of integration-competent, double-stranded DNA from the single-stranded viral RNA genome, combining both polymerizing and hydrolytic functions to synthesize approximately 20,000 phosphodiester bonds. Despite a wealth of biochemical studies, the manner whereby the enzyme adopts different orientations to coordinate its DNA polymerase and ribonuclease (RNase) H activities has remained elusive. Likewise, the lower processivity of HIV-1 RT raises the issue of polymerization site targeting, should the enzyme re-engage its nucleic acid substrate several hundred nucleotides from the primer terminus. Although X-ray crystallography has clearly contributed to our understanding of RT-containing nucleoprotein complexes, it provides a static picture, revealing few details regarding motion of the enzyme on the substrate. Recent development of site-specific footprinting and the application of single molecule spectroscopy have allowed us to follow individual steps in the reverse transcription process with significantly greater precision. Progress in these areas and the implications for investigational and established inhibitors that interfere with RT motion on nucleic acid is reviewed here.

Keywords

retroviruses; reverse transcriptase; ribonuclease H; translocational equilibrium; single molecule spectroscopy

Introduction

Almost 40 years have elapsed since the seminal discovery by Baltimore [1] and Temin and Mizutani [2] of an activity in retroviral particles catalyzing synthesis of DNA from an RNA template. Appropriately coined *reverse* transcriptase (RT), this enzyme mediates synthesis of integration-competent double-stranded DNA from the (+) strand RNA genome of the invading virus, using a combination of both RNA- and DNA-dependent DNA synthesis. An additional

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ribonuclease H (RNase H) activity associated with the same enzyme [3] removes RNA from the RNA/DNA replication intermediate to make nascent (–) strand DNA available as template for (+) strand DNA synthesis. During these events, the replication complex is transferred within an RNA template, or between templates of the diploid RNA genome. In human immunodeficiency virus (HIV), the final product of DNA synthesis is duplex DNA from which the RNA primers of (–) and (+) strand synthesis (tRNA^{Lys,3} and the polypurine tract (PPT), respectively) have been excised, but which contains a (+) strand discontinuity, reflecting cessation of synthesis at the central termination sequence (Figure 1) [4]. A detailed mechanistic description of the steps involved in proviral DNA synthesis can be found elsewhere [5], but for the purpose of this review, intimate cross-talk between RT and its conformationally-distinct nucleic acid substrates is clearly necessary to orchestrate such events.

A major advance in understanding reverse transcription at the molecular level has been the availability of structures of HIV-1 RT as apo-enzyme [6], in binary complexes with a nonnucleoside inhibitor of DNA synthesis [7] or duplex DNA [8] and in a ternary complex with duplex DNA and the incoming dNTP [9]. Although complemented by a variety of chemical and enzymatic probing methods [10,11], such studies provide an picture of a “static” enzyme, revealing little information on the process of translocation, i.e., the stepwise movement of the enzyme during DNA synthesis. Furthermore, specific steps during replication require the primer terminus to be alternately accommodated by catalytic centers located at either terminus of the polymerase, raising the issue of how enzyme orientation can be dictated by the structure of the nucleic acid substrate. Finally, lower processivity of HIV-1 RT poses a significant challenge in that, once dissociated enzyme re-binds, how does it access the polymerization site in an orientation competent to re-engage DNA synthesis? The answers to such questions have in part required implementation of new technologies to understand the process of both translocation and orientational dynamics of HIV-1 RT. Current understanding of these events is reviewed here and discussed in the context of both investigational and established RT inhibitors that interfere with RT motion.

1. Alternative Positioning of RT Determines Enzyme Function

During proviral DNA synthesis, RT encounters duplex RNA, RNA/DNA hybrids, and duplex DNA of varying lengths and sequence composition, and containing recessed 3' or 5' termini, 3' or 5' overhangs, nicks, gaps, and/or blunt ends (Figure 1). In this section, the means by which the enzyme differentially recognizes, binds, and processes these nucleic acid variants in order to convert viral RNA into a pre-integrative DNA intermediate is reviewed, with particular emphasis on RNase H-mediated processing of reverse transcription intermediates.

HIV-1 RT is a heterodimer of 66 and 51 kDa subunits (p66/p51). The larger of these houses an amino-terminal DNA polymerase domain comprised of fingers, palm and thumb subdomains, a central connection subdomain, and a carboxy-terminal RNase H domain, which collectively are supported by the smaller subunit. In co-crystal structures containing double-stranded DNA or an RNA/DNA hybrid, a bend of ~40° is imposed on the duplex ~6–8 bp from the primer terminus [8,9,12]. Although the DNA polymerase active site is located over the recessed 3' terminus of the primer strand in these structures, precise positioning of catalytic and other functionally important residues varies with the composition of the co-crystal. Conversely, the RNase H active center is located over the template strand 18 bp upstream, separated from the polymerase active center by ~70 Å. This mode of binding is also observed in chemical and enzymatic footprinting experiments on similar substrates [13,14], and is essential for DNA synthesis on either an RNA or DNA template.

The same binding mode is required for “polymerization dependent” RNase H activity, where the RNA template is partially hydrolyzed by RT during RNA-dependent DNA synthesis [15].

However, DNA synthesis and RNase H activities are not temporally coordinated under these conditions [16,17]. Hydrolysis is irregular, occurring in a pattern dependent upon RNA sequence and structure, and correlating in part with pausing of the enzyme during DNA synthesis [16,18]. At pause sites, or in the absence of DNA synthesis (which can be mimicked *in vitro* by excluding dNTPs from the reaction), cleavage of the RNA strand occurs 15–20 nt from the recessed 3' primer terminus. These cuts are referred to as "3'-directed", being largely dictated by polymerase domain binding at the primer terminus, and are consistent with the active site separation observed in X-ray crystal structures [12,15,17,19–21]

As a consequence of partial cleavage that occurs during RNA dependent DNA synthesis, RNA fragments of varying length remain hybridized to nascent DNA. These fragments, flanked by nicks and/or gaps of varying size, may be further processed by "polymerization independent" RNase H activity *via* "5'-directed" [18,19,22] or "internal" cleavage [23–27]. Within these partially hybrid substrates, no recessed DNA 3' (or 5') termini are available for RT to recognize and/or bind. Instead, in the case of 5'-directed cleavage, the polymerase active site localizes over the DNA strand opposite the recessed RNA 5' terminus, placing the RNase H active site for cleavage of the RNA strand 13–19 bp away. In contrast, hydrolytic events that occur at positions well removed from recessed termini of either strand are referred to as internal cleavages. In these cases, RNA sequence in the vicinity of potential cleavage sites is an important determinant of cleavage specificity and/or efficiency. Internal RNase H cleavage sequence preferences are summarized elsewhere [28]. Although 5'-directed and internal cleavage are less efficient than 3' directed cleavage, all modes of hydrolysis are essential for removing the viral genome during and following (–) strand DNA synthesis.

Specialized hydrolytic events necessary for completion of proviral DNA synthesis require selection and removal of the (–) and (+) strand primers (Figure 1). In a 3'-directed cleavage event, the tRNA^{Lys3} primer is incompletely removed from the (–) strand DNA template by RT-associated RNase H following synthesis of (+) strand strong stop DNA, i.e., a single 5'-terminal ribonucleotide remains attached to (–) strand DNA following cleavage [29]. Processing of the (+) strand primer is considerably more complex, but no less precise ([30]). Following (–) strand DNA synthesis, two purine-rich segments of the HIV RNA genome, designated the central and 3' polypurine tracts (c- and 3'-PPTs, respectively) are hydrolyzed at their 3' and 5' termini to generate the primers for (+) strand DNA synthesis [4,31,32]. Not only are these cleavage events precise, occurring at the rG-rA junction at the PPT 3'-termini, but resistance to internal hydrolysis is a consistent feature of these retroviral elements [33–35]. The critical 3'-terminal cleavage event, which defines the initiation site for (+) strand synthesis, is consistent with the rules and preferences governing both 5'-directed and internal cleavage. However, there is increasing evidence that PPT-containing RNA/DNA hybrids possess unique structural features that direct not only 3'-terminal cleavage [12,36–44], but also promote (+) strand initiation [35,44,45]. Initiation of (+) strand synthesis occurs after the 5' ribonucleotides immediately 3' to the PPT have been "cleared" by RT-associated RNase H, and requires reorientation of the enzyme on the hybrid duplex (see below). After incorporating 12 nt of DNA into the nascent (+) strand, RT again assumes an RNase H role to remove the PPT [46], thus ensuring the appropriate 5' LTR terminal sequence for recognition by the viral integration machinery. The enzyme dynamics required to catalyze these events are discussed in a subsequent section.

In exploring the interplay between the DNA polymerase and RNase H activities of RT and their various substrates, an issue that has recently received attention has been the ability of the DNA polymerase and the RNase H active sites of RT to simultaneously engage a hybrid substrate when bound in DNA synthesis/3'-directed RNase H cleavage mode. Unfortunately, conclusions derived from existing X-ray crystal structures are inconclusive in this respect. In all liganded structures to date, the DNA polymerase active center is engaged at the primer 3'

terminus while the RNase H active site is not appropriately positioned over the scissile phosphate in the template strand [8,9,12], suggesting that the two activities are mutually exclusive. However, all but one of these co-crystals contain double-stranded DNA, which is not a substrate for RNase H. Furthermore, in the exception [12], RT is positioned for internal cleavage of a PPT/DNA hybrid, which would not normally be expected to occur, and the substrate itself is quite unusual relative to other RNA/DNA hybrids. A model generated by superposition of the latter structure with that of a human RNase H-RNA/DNA complex also suggests that the two activities are mutually exclusive [47], but suffers from the same limitations as its RT-PPT/DNA counterpart [12]. More recent biochemical studies have shown that locking the 3' terminus of the primer at the polymerase active site in a ternary complex or with the inhibitor foscarnet produces efficient RNase H cleavage, suggesting that HIV-1 RT can simultaneously engage its DNA/RNA substrate with both active sites [48,49].

2. Single Molecule Spectroscopy: Snapshots of RT Conformational Dynamics

The previous section illustrates that synthesis of duplex DNA from the single-stranded retroviral RNA genome is a highly orchestrated program of events. The dynamics whereby (i), nascent DNA is transferred within, or between, templates [20] (ii), the (+) strand PPT primers are accurately recognized by catalytic domains located at either RT terminus and [30] and (iii), DNA synthesis terminates at phased A-tracts in the center of the duplex DNA product [4], might best be described as “retroviral gymnastics”, requiring intimate communication between RT and its single-stranded, hybrid and duplex nucleic acid substrates. While X-ray crystallography has provided incisive insights into the structure of the HIV-1 apoenzyme [50] and co-crystals with nucleic acid [8,9] and DNA polymerase inhibitors [7,51], it fails explain how RT assumes alternative conformations required to catalyze these events. Our early finding that the 3' terminus of a PPT embedded within a large (~100bp) RNA/DNA hybrid is accurately created via RNase H activity, extended into (+) strand DNA at the DNA polymerase catalytic center, then precisely removed from nascent DNA [52], suggests pre-existing features of the RNA/DNA hybrid dictate whether RT adopts a polymerizing or hydrolytic orientation [39]. An equally important issue is how HIV-1 RT, a polymerase with moderate processivity, re-engages in DNA synthesis, should it dissociate from the nascent template/primer. This requires mechanisms that not only allow targeting of the polymerization site, but also in an orientation competent to re-engage in DNA synthesis.

Since these processes are not readily amenable to examination by ensemble-based strategies, we turned our attention to examining the interaction of HIV-1 RT with nucleic acid by single molecule spectroscopy [36,53]. This strategy, outlined in Figure 2 (a), required placing a FRET donor (Cy3) at the N-terminal DNA polymerase or C-terminal RNase H domain RT and an acceptor (Cy5) onto a surface-immobilized nucleic acid duplex. An important validation of a FRET approach was demonstrating that modified enzyme could extend the primer of an immobilized DNA duplex despite extensive mutagenesis and covalent linkage of the FRET donor [36]. Since the N- and C-termini of HIV-1 RT are separated by ~100Å, enzyme orientation could be conveniently inferred from the strength of the FRET signal. Immobilizing the interacting complexes allowed conformational changes to be followed as a function of time and extraction of kinetic information.

HIV-1 RT orientation is determined by the RNA content of the primer

As would be predicted from X-ray crystallographic and biochemical studies, the high FRET signal obtained from C-terminally-labeled RT bound to a 50-nt DNA template/19-nt DNA primer (Figure 2 (b) *upper*) indicated a DNA synthesis-competent binding mode, placing the catalytic center of the DNA polymerase domain over the primer 3' terminus. In contrast, the low FRET signal obtained when an RNA primer of equivalent sequence was introduced (Figure 2 (b), *middle*) indicated a binding mode positioning the C-terminal RNase H domain in the

vicinity of the primer terminus. Moreover, when a Cy5-labeled RNA template was introduced, RT orientation still responded to the nature of the primer (Figure 2 (b), *right*). In support of our single molecule spectroscopy study, Palaniappan et al. [54] and De Stefano et al. [19] showed that on RNA/DNA hybrids on which both the 5' and 3' termini of the RNA strand are recessed, the 5' RNA terminus plays a dominant role in determining both the position and orientation of the enzyme.

Substituting chimeric RNA-DNA primers whose RNA content progressively increased from the 5' terminus allowed us to examine features of RNA/DNA hybrids that contributed to alteration in enzyme orientation (Figure 2 (c)). This strategy indicated that as few as two ribonucleotides at the primer 5' terminus (2R-17D) sufficed to initiate enzyme re-orientation, and that the process was virtually complete on a duplex containing a 5R-14D primer. The effect of a 5R-14D primer was particularly significant since the 14 bp DNA duplex portion of this substrate is almost sufficient to span the two catalytic centers. Although speculative, one clue to orientational bias may come from the co-crystal of HIV-1 RT and a PPT-containing RNA/DNA hybrid [8, 9, 12], which indicates that p66 residues Glu89, Gln91, Cys280 and Ala284 contact the ribose 2'-OH at several positions near the RNA 5' terminus. Since the equivalent contacts would be absent with duplex DNA [8, 9], additional hydrogen bonding afforded by the RNA strand may be sufficient to stabilize an orientation where its 5' terminus is accommodated within the DNA polymerase catalytic center, i.e., the primer is effectively recognized as the template. If correct, the orientational bias of HIV-1 RT on hybrid duplexes might be altered or eliminated by (i) replacing ribose 2' OH groups near the primer 5' terminus with 2' O-methyl or weakly H-bonding 2' F moieties, or (ii) by mutating residues involved in contacting this portion of the RNA strand to amino acids incapable of forming the necessary hydrogen bonds.

The “flips” and “flops” of polypurine tract recognition

(+) strand DNA synthesis in retroviruses and LTR-containing retrotransposons represents an unusual scenario where the RNA primer terminus must be accurately recognized by both the DNA polymerase and RNase H catalytic centers of the cognate RT [46]. In order to examine the dynamics of PPT processing, enzyme orientation was determined on RNA/DNA hybrids mimicking different steps in (+) strand synthesis (Figure 3). On an all-RNA primer whose 3' terminus was extended by two ribonucleotides beyond the PPTΔU3 cleavage junction (PPTr2), RT bound predominantly in an orientation positioning the RNase H domain for cleavage at this junction (Figure 3 (a), *left*). This result agrees with prior biochemical studies, as well as our expectation of what would occur at this stage of viral DNA synthesis. However, removing the two 3' ribonucleotides to make the PPT 3' terminus available for (+) strand priming, induced a re-distribution of enzyme orientation such that a significant fraction assumed a polymerization-competent orientation, despite the all-RNA nature of the primer (Figure 3 (a), *center*). Extending the PPT by two deoxynucleotides (PPTD2) likewise allowed RT to assume either a polymerization or cleavage orientation over the PPTΔU3 junction (Figure 3 (a), *right*), the latter of which is suitable for removal of the plus-strand primer once DNA synthesis has been initiated. The ability of RT to assume two distinct orientations on the PPT thus provides a mechanism whereby the (+) strand primer can be created, extended, and then precisely removed from nascent DNA.

To investigate structural rearrangements that might be required to promote a particular orientation, enzyme binding to a hybrid containing a chain-terminated PPTD2 primer was examined in the presence of the incoming dNTP, which would be predicted to establish a stable ternary complex [9]. Under these conditions, Figure 3 (b) shows that the presence of the incoming dNTP strongly favors a polymerization-competent binding mode in a concentration dependent manner. Conversely, on the same substrate, the nonnucleoside RT inhibitor

(NNRTI) nevirapine increases the frequency with which enzyme binds in the opposite, i.e., RNase H-competent, mode (Figure 3 (c)). Such reversal of enzyme orientation might explain why NNRTIs potently and preferentially inhibit initiation of HIV-1 (+) strand DNA synthesis [55]. Rather than the NNRTI acting directly on the chemical step of DNA synthesis, we interpret the FRET data as reflecting an enzyme bound in an orientation incompatible with DNA synthesis.

Structurally, these two ligands would be predicted to exert opposite effects on RT conformation at and near the DNA polymerase active site. While dNTP binding tightens the clamp of the p66 fingers and thumb subdomains around nucleic acid in a ternary complex [9], nevirapine (and other NNRTIs) occupy a hydrophobic pocket located at the base of the p66 thumb, with the consequence of loosening the grip on DNA.

Figures 3 (d) and (e) show representative FRET time traces for the ternary complex and RT/PPT/nevirapine complex, respectively. As might be expected ternary complex formation is reflected by a stable FRET trace (Figure 3 (d)). In contrast, RT bound to the PPT in the presence of nevirapine exhibited multiple transitions, or “flips” between low and high FRET states, indicating it spontaneously alternates its orientation *while continuously associated with the substrate* (Figure 3 (e)). In view of structural studies with RT/NNRTI complexes [56–59], these data suggests a potential pathway for spontaneously assuming alternate orientations that requires relaxation of the fingers-thumb grip. In addition to being required to re-orient on the PPT, data of the following section illustrates conditions where spontaneous re-orientation occurs in the absence of an NNRTI and while RT remains associated with the substrate.

Bringing it all together - flipping, sliding and polymerization site targeting

Compared with its cellular counterparts, HIV-1 RT is considerably less processive [31,60], frequently dissociating from nucleic acid during DNA synthesis. This property raises the issue of how the polymerization site is targeted on re-binding, i.e., does RT only bind at recessed DNA 3' termini, or can it bind elsewhere on the duplex and migrate to the site where DNA synthesis is to resume? Secondly, assuming that duplex nucleic acid confers no orientational bias on RT, how does the enzyme account for the possibility of accessing the polymerization site in the incorrect (RNase H) orientation?

Examining the distribution of HIV-1 RT on longer RNA/DNA hybrids and DNA duplexes by single molecule spectroscopy provided insights into how such events can be accomplished. Data of Figure 2 (b) and (c) was derived from substrates whose duplex portion was 19 bp, in order to exclude the possibility of multiple enzymes bound to a single substrate. The experiment of Figure 4 (a) follows the interaction of Cy3, RNase H-labeled RT with a 38 bp RNA/DNA hybrid, indicating a bimodal distribution of enzyme binding. Since a single binding event is under investigation, such data suggested that for ~75% of its “residency time”, RT is positioned with its DNA polymerase domain over the DNA primer 3' terminus, but that it can also disengage and relocate, or slide, to the opposite end of the duplex. Equivalent data were obtained with duplex DNA (*data not shown*), indicating that sliding is not exclusive to RNA/DNA hybrids. Furthermore, the end-binding states indicative of enzyme sliding predict that the two FRET peaks would be further separated as the duplex portion of the nucleic acid substrate was increased, which was experimentally confirmed with a 56-bp hybrid (*data not shown*). Since sliding presumably reflects opening and closing of the “grip” imposed by the p66 fingers and thumb subdomains on nucleic acid, this should respond to small molecules that affect RT architecture. Figure 4 (b) illustrates that establishing a ternary complex by including the incoming dNTP and a dideoxy-terminated primer enriches the low FRET binding mode by sequestering RT at the primer terminus. In contrast, nevirapine, which loosens the grip on nucleic acid, induces FRET signals of equal strength, indicating that the enzyme/NNRTI complex freely slides between the ends of the duplex (Figure 4 (c)). The capacity of

nevirapine to disengage the replicating enzyme from the polymerization site may be a second and important feature of this class of anti-RT drugs.

Sliding on the nucleic acid duplex provides a plausible mechanism whereby the replicating enzyme, having dissociated from its substrate, employs one dimensional diffusion to re-locate the polymerization site, as has been proposed for target searching by transcription factors, RNA polymerase and DNA repair enzymes [62–66]. However, once the polymerization site is accessed, it does not address how an incorrectly-oriented enzyme assumes a polymerization-competent orientation. Conceivably, enzyme arriving at the polymerization site incorrectly oriented may simply dissociate, and only when correctly-oriented to re-engage DNA synthesis does it remain stably bound. An alternative scenario is provided by the data of Figure 4 (d) and (e), where the interaction of RNase H-labeled RT with a 550b bp DNA duplex containing Cy5 at one terminus was monitored. Under these labeling conditions, the ability to re-engage the polymerization site in the absence of one-dimensional diffusion would predict a low FRET value immediately on enzyme binding. In contrast most binding events initiated with a FRET value of 0 and reached 0.3 only after a finite time delay (Figure 4 (d), *left*), suggesting that HIV-1 RT contacted the DNA duplex distal to the polymerization site and subsequently diffused to the primer terminus in an orientation competent to re-engage in DNA synthesis. Such a binding procedure increases the efficiency with which the polymerization site is accessed on long duplexes where the primer terminus constitutes a miniscule fraction of the total substrate.

Since re-association of HIV-1 RT with its nucleic acid substrate could occur at multiple positions, the lack of directional bias could result in accessing the polymerization site in the opposite orientation, i.e. positioning the C-terminal RNase H domain in the vicinity of the primer 3' terminus. Under such conditions, the short time delay following binding would be followed by a high FRET state. In approximately 50% of the binding events monitored [53], this was indeed the case, indicated by the FRET trace of Figure 4 (d) (*right*). This high FRET state was, however, transient and rapidly reverted to the low FRET state, indicating that RT spontaneously “flipped” into a polymerization-competent orientation. As evidenced with earlier studies with nevirapine, re-orientation to assume a polymerization-competent configuration occurred without dissociation of RT from the DNA duplex. The two scenarios are represented schematically in Figure 4 (e), supporting the notion that a combination of sliding and flipping provides an efficient means of efficiently accessing and productively initiating DNA synthesis from the polymerization target site.

Pushing to its limits: the dynamics of strand displacement synthesis

RNA and DNA strand displacement synthesis are pre-requisites for HIV RT to (i), polymerize through intra-strand RNA duplexes such as the U5-IR and TAR loops following initiation of (–) DNA synthesis (ii), remove residual RNA during (+) strand synthesis (iii), displace cPPT-initiated (+) DNA to create the “central termination sequence” and (iv), duplicate the viral LTRs (Figure 1). Hybridizing Cy3- and Cy5-labeled primer and non-template strands to a single template allowed the design of model substrates to investigate the dynamics of strand displacement synthesis [53]. While RNA strand displacement was demonstrated upon limited DNA synthesis, repetitive transitions between two FRET states indicated re-annealing of the displaced RNA strand and displacement of nascent DNA. One explanation for this result was enzyme shuttling, i.e. RT disengaged from the polymerization site and re-located to the distal end of the duplex, which could be demonstrated experimentally. Once the overlap between the extended DNA primer and the non-template RNA exceeded 4 nucleotides, RT failed to access the polymerization site, most likely because the energetics of displacing the RNA strand were no longer favorable. Restricted access to the polymerization site was not observed when the non-template RNA was substituted with DNA, presumably reflecting equal stability of the

nascent and displaced DNA duplexes. Displacement synthesis over the entire length of the non-template strand was, however, accompanied by pausing at several positions, the mechanistic basis for which is under investigation.

Single molecule spectroscopy thus illustrates HIV-1 RT to be a remarkably versatile enzyme capable of sliding and spontaneously alternating orientation in order to access the polymerization site in a manner competent to re-engage in DNA synthesis. The demonstration that nevirapine stimulates these events offered new and alternative insights into the mechanism of NNRTI inhibition that were inaccessible through ensemble-based techniques. Finally, additional aspects of reverse transcription, including features of the replication intermediate that promote DNA strand transfer and recombination, as well as the role of the viral nucleocapsid protein, clearly lend themselves to single molecule spectroscopy to unravel the acrobatics of this multifunctional enzyme.

3. Nucleotide Incorporation and RT Translocation

Initiation of DNA synthesis requires that RT binds duplex nucleic acid and recognize the 3' terminus of the primer strand. Once this occurs, the enzyme faces a variety of challenges, including incorporating the incoming dNTP and advancing to the next template position before the complex dissociates. Co-crystals of HIV-1 RT containing primer/template show that the nucleotide binding site (N-site) is generally accessible, while the primer 3' terminus occludes the adjacent priming site (P-site). In this configuration, the enzyme can accommodate the incoming dNTP, which is trapped through closure of the fingers subdomain [9]. Catalysis requires the presence of Mg^{2+} ions that are coordinated by highly conserved aspartic acid residues. A general model suggests the involvement two divalent metal ions, designated A and B. Metal ion A supports nucleophilic attack of the 3'-OH group of the primer on the α -phosphate of the bound nucleotide, while metal ion B is likely to be involved in the release of pyrophosphate (PPi) [67]. Deprotonation of the 3'-OH group and protonation of the PPi leaving group may be regulated by active site residues [68,69]. The low affinity for PPi appears to drive the reaction forward. Pyrophosphorolysis is negligible, unless the forward reaction is blocked following incorporation of a chain-terminating nucleotide [70]. Binding of the next nucleotide requires the polymerase to move a single position further downstream, which brings the primer 3-terminus back to the P-site. Such motion of the polymerase relative to its nucleic acid substrate is defined as translocation (Figure 5). RT translocation is crucial for the entire polymerization process as it provides the link between successive cycles of nucleotide incorporation.

Translocation of the polymerase appears to be at least as fast as the nucleotidyl transfer step, which makes it difficult to define this step kinetically [17]. Pre-steady state kinetic analyses have revealed a biphasic burst of product formation during single nucleotide incorporation by HIV-1 RT [71,72]. Based on kinetic data with short, defined primer/template substrates, it has been suggested that initial formation of the RT-nucleic acid complex can result in three different populations, namely (i) dead-end complexes, (ii) polymerase incompetent complexes that can be converted into polymerase competent complexes, and (iii) polymerase competent complexes that allow instantaneous binding and incorporation of the nucleotide substrate [72]. Moreover, the biphasic dissociation kinetics reported by Ignatov et al. [73] point to the existence of different forms of nucleoprotein complex. Although the nature of the complex is not further defined in these studies, it is conceivable that interconvertible forms of RT-nucleic acid complexes represent pre- and post-translocational states. FRET-based single-molecule experiments also suggest to the existence of two complexes that differ by a 5 Å shift in the position of the nucleic acid [74], possibly also indicative of pre- and post-translocational configurations. The presence of the incoming dNTP provides conditions favoring the latter (longer distance between the dyes), while the presence of PPi promotes conditions favoring

the former (shorter distance between the dyes), although pyrophosphorolysis cannot be excluded under the assay conditions.

Ding et al. [75] suggested that structural elements of HIV-1 RT interacting with nucleic acid could act as modular elements to form a “translocation track”. The movement of these elements against each other could break and reform contacts with nucleic acid, which ultimately induces translocation of the enzyme relative to its bound substrate. What, however, is the driving force for translocation? Two alternative mechanisms have been proposed. An active mechanism, designated the “powerstroke” mechanism, postulates that dNTP hydrolysis and/or the release of PPi provide the energy for translocation [76]. In contrast, a passive mechanism postulates that translocation is driven by random thermal energy in the form of Brownian motion, and nucleotide hydrolysis is not required [77]. In the latter model, the polymerase can freely oscillate between pre- and post-translocational configurations and the nucleotide substrate traps the complex post-translocation, which ultimately drives the reaction forward.

A detailed version of an active mechanism of RT translocation is based on the structures of binary complexes that exist either pre- or post-translocation and contain 3'-azido-3'-deoxythymidine monophosphate (AZT-MP) at the primer 3'-end terminus [78]. A comparison of these structures with the ternary complex suggested that nucleotide binding is associated with displacement of the highly conserved YMDD motif that constitutes part of the active site. The motion was compared with the “loading of a springboard”. The “release of the springboard”, following catalysis may provide part of the energy required for translocation.

A passive model of RT translocation is supported by several biochemical studies. Site-specific footprinting techniques have been utilized to distinguish between pre- and post-translocated complexes in solution [79]. These methods are based on specific RT-mediated cuts on nucleic acid, as opposed to classical footprinting approaches that are based on nucleic acid protection. Treatment of RT-DNA/DNA complexes with Fe^{2+} ions produces major cuts at template positions -17 or -18. Fe^{2+} ions were shown to bind at or in close proximity to the RNase H active center, and oxidation of the bound metal ions yields a local source of hydroxyl radicals that cleave the template in site-specific fashion. An alternative footprinting approach involves a metal-free source of hydroxyl radicals. Treatment of RT-DNA/DNA complexes with potassium peroxyntirite yields hyper-reactive cleavage at template position -7 or -8 (Figure 6). Here the cut is mediated through the sulphur of Cys280 that, in the large subunit of HIV-1 RT, lies in the vicinity of the template [13].

The ratio between adjacent cuts at positions -7 and -8 (or -17 and -18) depends on several parameters, including the sequence of the primer/template duplex. Certain sequences yield footprints with specific cuts at positions -8, and -18, respectively. Introducing the incoming dNTP to such a complex can shift cleavage toward positions -7 and -17, respectively, suggesting the enzyme has moved a single template position further downstream. This movement is not a consequence of nucleotide incorporation, because the primer was terminated with a nucleotide analogue lacking the 3'-OH group. The data rather show that the incoming dNTP traps the post-translocational state. In other words, the cut at position -8 (or -18) is indicative for the pre-translocational state in which the primer terminus occupies the N-site, while -7 (or -17) cleavage indicates a the post-translocational state where the primer terminus occupies the P-site and the nucleotide substrate is bound to the N-site. DNase I footprinting and exonuclease protection assays are in agreement with these findings [80], providing strong evidence for the existence of a translocational equilibrium. RT may indeed rapidly oscillate between the two states and the incoming nucleotide can act like a pawl of a ratchet and traps the post-translocational complex. Nucleotide hydrolysis is not required for RT translocation. Thermal energy in form of Brownian motion appears to be sufficient to trigger the motion of RT; hence, this model of polymerase translocation is generally referred to as “Brownian

ratchet” model [81]. Moreover, this model is consistent with the notion that RT slides along its nucleic acid substrate in the absence of DNA synthesis. The combined processes of sliding long distances to establish productive interaction between the polymerase active site and the 3' terminus of the primer, and translocation over short distances to ensure successive nucleotide incorporation events appears to be driven by Brownian motion.

4. Effects of antiviral drugs on the conformational dynamics of HIV-1 RT

Nucleoside RT Inhibitors (NRTIs)

NRTIs mimic the natural substrates of DNA synthesis and constitute the backbone of HIV treatment regimens. Currently, 8 NRTIs have received FDA approval, namely zidovudine (AZT), lamivudine (3TC), emtricitabine (FTC), didanosine (ddI), abacavir (ABC), the nucleotide analog tenofovir (TFV), stavudine (d4T), and although no longer in clinical use, zalcitabine (ddC). These inhibitors are administered as prodrugs to facilitate cellular entry and are subsequently phosphorylated by host enzymes into an active triphosphate (TP) form that is incorporated into viral DNA by RT. This results in chain termination due to the absence of a deoxyribose-associated 3' OH – a structural feature common to all clinically approved NRTIs. Otherwise, NRTI structures are quite diverse, consequently producing variable clinical results, and ultimately invoking differing mechanisms of drug resistance [82].

Although incorporation of a chain terminator into the primer prevents further DNA synthesis, RT may translocate along the substrate and bind the next incoming nucleotide, depending on whether the NRTI creates a steric barrier to doing so. For example, when a dideoxy-chain terminator has been incorporated, the chemical moiety at the primer 3' position is quite small. Consequently, RT retains the capacity to translocate along the chain-terminated primer and bind the next nucleotide, and the p66 fingers will adopt a closed conformation to stabilize the ternary complex. However, the enzyme will be unable to catalyze phosphodiester bond synthesis, resulting in a stable, “dead-end” complex exemplified in the crystal structures of RT with dsDNA and either dTTP or tenofovir diphosphates [9,83].

Conversely, ternary complexes wherein the primer strand is terminated with NRTIs containing an 3' azido group are not stable, suggesting that steric barriers produced by the analog inhibit translocation and subsequent nucleotide binding. This notion has been confirmed by X-ray crystallographic and molecular modeling studies, which predict that a steric conflict between the incoming nucleotide and the azido group of AZT at the primer terminus limits the complex to assuming an exclusively pre-translocation state [78,84,85]. Footprinting studies revealed that chain termination with tenofovir yields an intermediate result, in which translocation is inhibited, but not to the extent observed with AZT [79,86]. Classical NRTI resistance-conferring mutations that are clustered around the N-site do not appear to affect the conformational dynamics of HIV-1 RT. However, it has recently been shown that distant mutations in the connection and RNase H domain of HIV-1 RT can reduce susceptibility to AZT [87]. Some of these mutations, including Asn348Ile, selectively diminish binding in polymerization independent, RNase H competent complexes [88]. As a consequence, degradation of the RNA template is delayed, which provides more time for removal of the incorporated chain terminator by pyrophosphorolysis. Connection domain mutations often co-emerge with classical NRTI resistance mutations that accelerate the ATP-dependent excision of AZT [89].

Delayed chain terminators (DCTs) represent a class of NRTIs that retain a chemically active 3'-OH. Extension of the nascent primer occurs even after they are incorporated. However, DNA synthesis is eventually impaired, presumably due to DCT-induced steric interference between template/primer and portions of the nucleic acid binding cleft in the immediate vicinity of the polymerase active site. Moreover, because the DCT is located several nucleotides from

the 3' terminus of the nascent DNA when the inhibitory effect is manifest, its excision essentially becomes impossible. North methanocarba-nucleotide triphosphates (N-MCN-TPs), for example, possess a constrained pseudosugar ring, and block DNA extension *in vitro* through a delayed chain termination mechanism [90–92]. However, because these inhibitors were constrained in the North or 2'-endo conformation, they could not be phosphorylated by cellular kinases and therefore possessed no antiviral activity [92]. Another delayed chain terminator, entecavir (ETV), is a nucleoside analog inhibitor of hepatitis B virus RT that also weakly inhibits HIV-1 RT. ETV was shown to block DNA synthesis by both enzymes, inducing strong pausing three nucleotides after its incorporation into the primer strand [93,94]. ETV-MP is efficiently excised by RT when present at the 3' terminus; however, delayed chain-termination protects the inhibitor from excision also in the context of major NRTI resistance conferring mutations [94]. RNase H mapping experiments revealed exclusively polymerization-independent cuts, suggesting that the 3' terminus of the primer is repelled from the polymerase active site [94]. Thus, it appears that the incorporated delayed chain-terminator can affect the equilibrium between sliding and primer recognition. The polymerization-competent complex may not be stabilized and trapped, and as a consequence, RT continues to move on its nucleic acid substrate.

Phosphonoformic Acid (PFA)

It is tempting to ask whether other classes of RT inhibitor likewise affect the translocational equilibrium and/or polymerase sliding over longer distances. The PPI-analogue PFA shows a broad spectrum of antiviral activities against various members of the *herpesviridae* and *retroviridae*. The inhibitor is used in the clinic to treat infection with herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2), human cytomegalovirus (HCMV), and other related herpesviruses. Despite problems associated with its clinical use, including adverse effects and poor oral bioavailability, PFA has sometimes been administered to patients infected with multi-drug resistant HIV-1 variants when other treatment options were unavailable [95]. The beneficial effects of this drug have been primarily associated with its resistance profile. With few exceptions, mutations that confer decreased susceptibility to NRTIs and NNRTIs do not affect susceptibility to PFA. Conversely, mutations associated with PFA resistance were shown to increase susceptibility to AZT [96].

“Hot spots” for PFA inhibition are strongly associated with sequences that show a bias toward pre-translocation [97]. In contrast, sequences showing a bias toward the post-translocational state are resistant to PFA inhibition. Footprinting experiments and binding studies confirmed that PFA traps the pre-translocated complex, strengthening the notion that RT freely oscillates between pre- and post-translocated positions and both complexes can be targeted during active DNA synthesis (Figure 7). PFA binding is increased with increasing concentrations of divalent metal ion, suggesting it occupies the polymerase active site. Binding of PFA and the natural nucleotide substrate are therefore mutually exclusive. The non-competitive mode of inhibition often measured under steady-state conditions reflects increased stability of the product complex, which diminishes the turnover of the reaction.

Nucleoside-competing RT Inhibitors (NcRTIs)

Indolopyridones represent a newly-discovered class of RT inhibitors with yet another unique mechanism of action [98,99]. The prototype compound, INDOPY-1, is active against NNRTI-resistant HIV strains. Like PFA and the natural nucleotide substrate, respectively, INDOPY-1 can bind to and stabilize RT-DNA/DNA complexes. However, footprinting experiments and binding studies revealed that the complex with the inhibitor is trapped in its post-translocational state, although the structure of the inhibitor differs significantly from natural nucleotide substrates. Binding of INDOPY-1 depends on the chemical nature of the ultimate base pair at the primer 3' terminus and not the chemical nature of the templated base that is engaged in

classic base-pairing. INDOPY-1 binds preferentially following pyrimidines (thymidines > cytidines).

Steady-state kinetic analysis with homopolymeric substrates revealed competitive, or mixed-type (competitive/non-competitive) inhibition, respectively. Together, these findings suggest that the binding site for indolopyridones and nucleotide substrates can at least partially overlap. The resistance profile of INDOPY-1 provides further independent evidence for this notion. *In vitro* selection experiments and phenotypic susceptibility measurements with clinical isolates and constructs generated by site-directed mutagenesis suggest that most mutations associated with decreased susceptibility to INDOPY-1 are clustered around the dNTP binding site [100]. These mutations include the NRTI-associated mutations Met184Val and Tyr115Phe. In light of the combined data, this class of compound is referred to as nucleotide-competing RT inhibitors (NcRTIs).

Conclusions

Recent development of novel biochemical and biophysical techniques, including site-specific chemical footprinting and single molecule spectroscopy, have facilitated a detailed study of dynamic conformational changes associated with RT-nucleic acid complexes. The collective data of this review suggest that each of the various classes of RT inhibitors can interfere with RT in motion, which is largely driven by thermal energy. NNRTIs interfere with the orientation of HIV-1 RT on its nucleic acid substrate, and the chemical nature of NRTIs can affect the translocational equilibrium. The study of mechanisms of action associated with PFA and INDOPY-1 provides proof-of-principle that small molecule inhibitors can specifically target pre- and post-translocated complexes. Moreover, delayed chain-terminators appear to affect the equilibrium between sliding and primer recognition, and, in turn, establishment of a translocational equilibrium. Together, these examples suggest that additional consideration is given to HIV RT dynamics in future drug discovery and development efforts.

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Figure 1.

HIV-1RT-catalyzed synthesis of double-stranded, integration-competent DNA (black) from the single-stranded viral RNA genome (grey). (-) strand DNA synthesis, initiated from tRNA_{Lys,3} bound to the primer binding site (PBS), proceeds to the RNA 5' terminus, copying repeat (R) and unique 5' (U5) sequences. Concomitant RNase H activity hydrolyzes the ensuing RNA/DNA hybrid, allowing complementary R sequences at the genome termini to promote transfer of nascent DNA to the RNA 3' terminus and continued DNA synthesis. Hydrolysis of the RNA/DNA replication intermediate continues, with the exception of the 3' and central PPT (cPPT), which prime (+) strand, DNA-dependent DNA synthesis up to, and including, 18 nucleotides of the tRNA primer, creating a complementary (+) strand PBS sequence. The PPT

and tRNA primers are excised, and PBS complementarity promotes a second strand transfer event, after which bidirectional DNA synthesis creates double stranded proviral DNA flanked by the hallmark long terminal repeat (LTR) sequences. The presence of a cPPT in HIV creates a “central flap” which is later processed by host-coded enzymes.

Figure 2.

Examining HIV-1 RT dynamics by single molecule spectroscopy. (a), *Upper*, HIV-1 RT is site-specifically labeled with the FRET donor Cy3 at its N-terminal fingers subdomain or C-terminal RNase H domain. *Lower*, Cy3-labeled RT interacts with surface-immobilized DNA containing the acceptor dye Cy5, and fluorescence of individual substrates is followed by total-internal-reflection fluorescence (TIRF) microscopy with alternating laser excitations at 532 and 635nm. 532-nm light selectively excites Cy3, and follows FRET between RT and Cy5-labelled DNA, while 635-nm light directly excites Cy5, verifying the presence of the nucleic-acid substrate and FRET acceptor. (b), Alternative orientations of HIV-1 RT on nucleic acid duplexes. FRET histograms are shown for RNase H-labeled RT bound to a 30-nt DNA/19-nt DNA duplex (*upper*), a 30-nt DNA/19-nt RNA hybrid of equivalent sequence (*middle*), and a 30-nt RNA template/19-nt DNA primer (*lower*). Cy5 was introduced near the 5' terminus of all templates. (c), FRET histograms of RNase H-labelled RT bound to Cy5-labeled DNA to which chimeric RNA/DNA primers were hybridized. RNA/DNA primer notations (R/D) are presented in a 5' – 3' orientation. Cy5 was introduced near the 5' terminus of all templates.

Figure 3.

HIV-1 RT adopts alternative orientations on the PPT. (a), FRET histograms derived from RNase H-labeled RT incubated with RNA/DNA hybrids whose PPT RNA primer was extended at its 3' terminus with two ribonucleotides (PPTTr2, *left*) or two deoxynucleotides (PPTD2, *right*) or lacking an extension (*middle*). (b), FRET time trace of HIV-1 RT bound to a chain-terminated PPTD2 substrate in the presence of dNTPs, establishing a stable ternary complex. (c), FRET time trace of HIV-1 RT bound to a PPTD2 substrate in the presence of nevirapine (NVP), indicating multiple transitions, or “flipping” between high and low FRET states. (d) Ternary complex formation promotes enzyme binding to the primer terminus in a polymerization orientation. Histogram of RT bound to PPT:dd2 hybrid (filled, grey) in the presence of 10 μ M (purple trace) and 1mM dTTP (cyan trace) (e), NNRTI binding promotes RT binding to the PPT 3' terminus in an RNase H orientation. Histograms of RT bound to PPT:d2 substrates in the absence (filled grey trace) presence of 10 μ M (red trace) and 100 μ M NVP (orange trace).

Figure 4.

HIV-1 RT sliding on an RNA/DNA hybrid. (a), RNase H-labeled RT (H) was bound to a 3'-labeled 38 bp RNA/DNA hybrid. In contrast to the single FRET peak obtained with shorter hybrids, low and high FRET peaks are observed. The former represents an enzyme whose DNA polymerase domain is positioned over the DNA primer (black) 3' terminus, and the latter one that has disengaged from the primer terminus and relocated to the far end of the hybrid. (b), Ternary complex formation eliminates sliding. RT binding to a duplex containing a chain-terminated primer (blue histogram) was examined in the presence of all four dNTPs (green trace) or the incoming dNTP at 200 μ M (orange trace) and 1 mM (red trace). (c), The NNRTI nevirapine (NVP) facilitates sliding. Sliding was examined on a 38-bp binary complex (blue histogram) in the presence of 20 and 100 μ M NVP (orange and red traces, respectively). (d) Sliding aids polymerization site targeting. RNase H labeled RT was incubated with a ~ 550-bp DNA duplex. Binding to the polymerization site is expected to give a FRET value of 0.3. *Left*, Donor and acceptor fluorescence (*upper*) and corresponding FRET time traces of a typical binding event (*lower*) show a time delay (t) between RT binding and its arrival at the polymerization site. *Right*, A binding event showing a transient 0.9 FRET state, suggesting RT reached the polymerization site in an RNase H orientation and subsequently flipped to the polymerization orientation. Yellow shading of the FRET time traces mark individual RT binding events. (e) Proposals for the alternative primer terminus search scenarios.

Figure 5.

Model of single cycle of nucleotide incorporation. Incorporation of a nucleoside monophosphate by nucleic acid polymerases involves substrate binding, a conformational change to trap the incoming dNTP, phosphodiester bond formation, pyrophosphate release and ultimately enzyme translocation to clear the nucleotide binding site. Positions of the P site (priming- or product-site) and N-site (nucleotide binding site or substrate site (S-site)) are depicted relative to template positions i and $i+1$. The former is complementary to the primer 3' terminus (green) and the latter represents the insertion site for the first nucleotide (red). See [81] for additional details

Figure 6.

Site-specific KOONO footprinting of pre- and post-translocation complexes containing HIV-1 RT. The duplex DNA substrate is indicated in (a), the primer of which is 3' terminated by incorporation of azidothymidine monophosphate (Z). Chemical footprinting was performed in the presence of increasing concentrations of dGTP, the incoming dNTP. Pre- and post-translocational states of HIV-1 RT correspond to KOONO-mediated cleavage of the template strand at positions -8 and -7, respectively. See [79] for additional details.

Figure 7.

Proposed model for foscarnet (PFA) binding to HIV-1 RT, mechanism of action, and drug resistance. Pre- and post-translocational states are in equilibrium. PFA is shown schematically as black circles in the pre-translocated complex. The nucleotide occupies the N-site in the post-translocated complex. PFA and the phosphates of the bound deoxynucleoside triphosphate (dNTP, blue circles) are shown in close proximity to the divalent metal ions (open circles). Additional details can be found in [97].