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Dynamics: the missing link between structure and function of the viral RNA-dependent RNA polymerase?

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The structural basis for nucleotide incorporation fidelity remains an open question for all nucleic acid polymerases. Addressing this question for the viral RNA-dependent RNA polymerase (RdRp) is of particular, practical significance because it is a determinant of sensitivity to antiviral nucleosides and may be a determinant of viral virulence. All polymerases are thought to employ the same catalytic mechanism, but the rate of nucleotide incorporation can vary substantially. Here we review some of the recent work with the RdRp that leads us to suggest that structure provides only a partial understanding of RdRp function and dynamics may be the missing link.

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Introduction

RNA viruses represent the largest class of existing and emerging human pathogens. Few vaccines have been licensed to prevent RNA virus infections, and only one broad-spectrum antiviral (ribavirin) has been licensed to treat RNA virus infections in humans. Unfortunately, this single antiviral agent is not effective against all RNA viruses [1]. Replication of the RNA virus genome is catalyzed by the virus-encoded RNA-dependent RNA polymerase (RdRp) [2]. Like other viral polymerases, the RdRp is an important target for the development of antiviral therapeutics. Rational design of RdRp-specific, nucleotide-based inhibitors and/or substrates would benefit from the knowledge of how catalytic efficiency and nucleotide specificity are engineered into the active site of the enzyme. Addressing these very fundamental issues regarding polymerase function is among the most active areas of polymerase enzymology. Herein we review some of the most recent studies of RdRp structure and

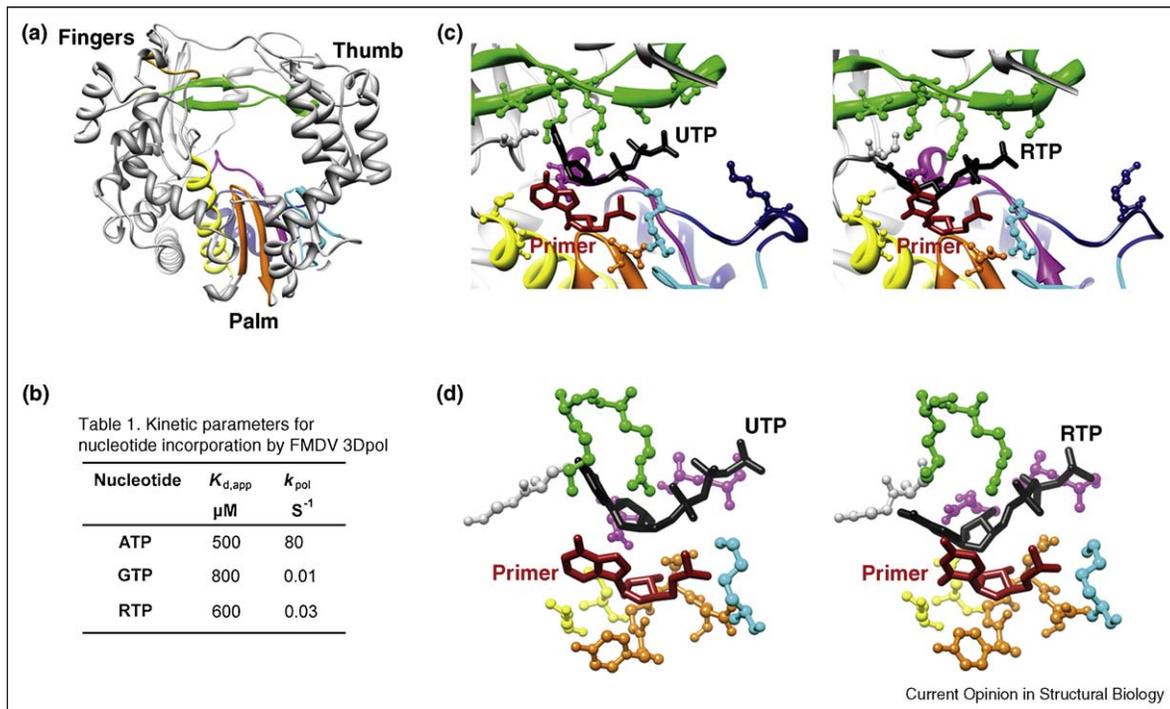
function. We conclude that existing gaps in our ability to connect RdRp structure to RdRp function may require investigations into RdRp dynamics.

Structures of the RdRp are unable to explain the reduced catalytic efficiency of antiviral nucleoside triphosphates

RdRps have a semi-classical structural organization that is often compared to a right hand (Figure 1a). The enzyme not only has the fingers, palm, and thumb subdomains found in other classes of nucleic acid polymerases but also has a fingertips subdomain that leads to the formation of a completely encircled active site. Seven conserved structural elements, termed motifs A–G, exist. Motifs A–E are located in the palm subdomain and contribute to aspects of nucleotide and nucleic acid binding, as well as catalysis. Motif C is the most well known motif as it contains the GDD sequence that is a hallmark of viral RdRps. The function of motif D is only now becoming clear and will be discussed below. Motifs F and G are located in the fingertips and fingers subdomains, respectively. Motif F contains basic amino acid residues that appear to be responsible for ground-state binding of the triphosphate moiety of the incoming nucleotide [2]. The function of motif G is not absolutely clear, although substitutions of residues in this motif inhibit polymerase activity [3].

RdRps have often been characterized as error-prone polymerases. However, it is now clear that the low fidelity is primarily due to the absence of proofreading exonuclease activity [4]. RNA viruses with genomes longer than 10 000 nt, coronaviruses, for example, may encode a proofreading activity [5••]. Transition mutations do occur with a frequency of 1 in 10 000, primarily due to GMP misincorporation opposite uridine (Figure 1b). RdRps do not distinguish correct nucleotides from incorrect nucleotides or nucleotide analogs, such as ribavirin triphosphate (RTP), at the binding step as the apparent dissociation constants ($K_{d,app}$) for all nucleotides are quite similar (Figure 1b), consistent with motif F interactions with the triphosphate governing nucleotide binding [4]. The primary fidelity checkpoint for the RdRp occurs after binding and represents a conformational change that has been attributed to the reorientation of the triphosphate into a position for efficient catalysis [4,6]. Changes in the equilibrium constant for this conformational-change step manifest as a change in the maximal rate constant for nucleotide addition (k_{pol}) (Figure 1b).

Figure 1



Limited flexibility observed of the RdRp during the catalytic cycle. RdRp from foot-and-mouth disease virus (FMDV) is employed here but is a representative for most viral RdRps [8**]. **(a)** FMDV RdRp in the absence of ligands. The conserved structural motifs are highlighted by different colors (motif A, magenta; motif B, yellow; motif C, orange; motif D, blue; motif E, cyan; motif F, green; and motif G, gold). **(b)** Kinetic parameters: $K_{d,app}$ and k_{pol} for correct (ATP), incorrect (GTP), and ambiguous (RTP) nucleotide incorporation catalyzed by FMDV RdRp. Parameters were obtained from Ref. [31]. **(c)** Close-up view of the RdRp active sites in the presence of ligands: RNA and UTP (left, pdb 2E9Z); RNA and RTP (right, pdb 2E9R). The 3'-terminal nucleotide of RNA primer is shown as red sticks; the nucleotide and the nucleotide analog are shown as black sticks. **(d)** Active site residues within 5 Å of the RNA terminus and incoming nucleotides are shown as balls and sticks.

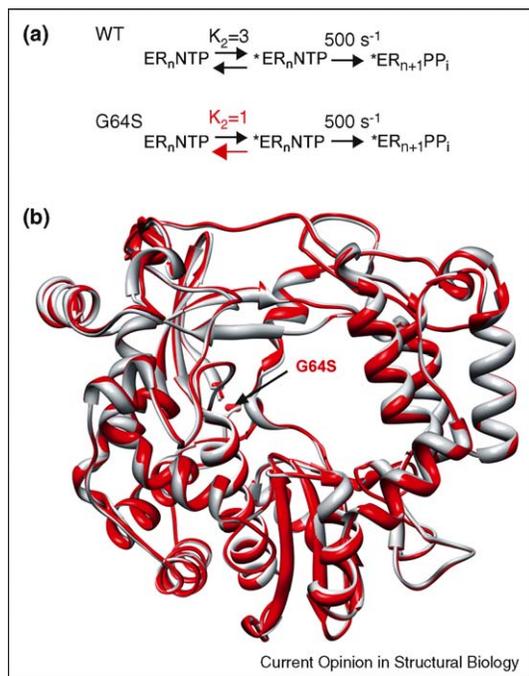
One of the most exciting advances in the field of RdRp enzymology recently was the solution of structures for the RdRp from foot-and-mouth disease virus (FMDV) in complex with primed template and various correct nucleotides and RTP [7,8**]. Because the fingertips of the RdRp constrain movement of the fingers subdomain relative to the thumb subdomain, reorganization of these subdomains did not occur as observed in polymerases that lack a fingertips subdomain [8**]. This observation suggests that subdomain movements are not responsible for rate-limiting, conformational-change steps observed by kinetic analysis of the single-nucleotide-addition cycle. The most substantial difference between a complex with a correct nucleotide (UTP in Figure 1c) relative to a complex with an inefficiently incorporated nucleotide analog (RTP in Figure 1c) was the position of residues, such as Lys-369, on motif D. The complexes are essentially superimposable when atoms involved in catalysis are interrogated (Figure 1d). As discussed below, Lys-369 (Lys-359 in poliovirus (PV) RdRp) of motif D is a dynamic element that influences the rate of catalysis. Structural studies may

fail to identify the basis of fidelity because they are unable to capture the trajectory of motif D during a catalytic cycle.

Remote sites control active site, fidelity checkpoints

Several surprises regarding the mechanistic basis for RdRp fidelity came from studies of PV that were aimed at identifying a high-fidelity polymerase by selecting for PV mutants that exhibit reduced sensitivity to ribavirin [9,10]. The first surprise was that a site remote from the catalytic center controlled fidelity. Gly-64 of the PV RdRp, a residue in the fingers subdomain, was changed to Ser. Gly-64 has an indirect connection to motif A in the active site; this motif contributes to interactions in the ribose-binding site and coordination of Mg^{2+} ions required for catalysis [9]. A second surprise was that this same mutant was isolated independently by two different laboratories, suggesting that few solutions may exist to development of resistance to this class of antiviral nucleosides, a distinct contrast to other classes of nucleoside-based RdRp inhibitors [11].

Figure 2



Structure is unable to reveal the basis for RdRp fidelity. **(a)** The conformational-change step (K_2) is used to alter fidelity of G64S PV RdRp [9]. **(b)** Crystal structure of the high-fidelity mutant G64S in PV-3Dpol is essentially identical to the WT (rmsd of 0.33 Å) [26*].

Mechanistic studies of the PV G64S polymerase revealed that this substitution decreased the equilibrium constant for the conformational-change step (K_2) preceding chemistry (Figure 2a) [9]. The more difficult it is for the enzyme to achieve the catalytically competent conformation, the more faithful the nucleotide-addition cycle will be. Structural studies of the G64S polymerase in complex with substrates have not been completed, but the structure of the unliganded enzyme provided no insight into changes in the organization of motif A as might have been predicted (Figure 2b). Because conformational changes appear to be altered by the G64S substitution, analysis of the dynamics during the nucleotide-addition cycle may be most insightful.

Biological studies of PV encoding the G64S substitution have shown that the fidelity of the PV RdRp appears to be important for pathogenesis. The ability for G64S PV to cause disease was attenuated in a very permissive mouse model [12*,13*]. The attenuated phenotype was shown to be a reflection of the reduced population diversity produced by a high-fidelity polymerase by using chemical mutagenesis to restore virulence to the G64S PV population [13*]. Importantly, G64S PV and related mutants confer long-term protection of mice from a lethal challenge with wild-type PV [14**]. Therefore, an understanding of how fidelity is engineered into

the RdRp structure has practical implication for the development of vaccines.

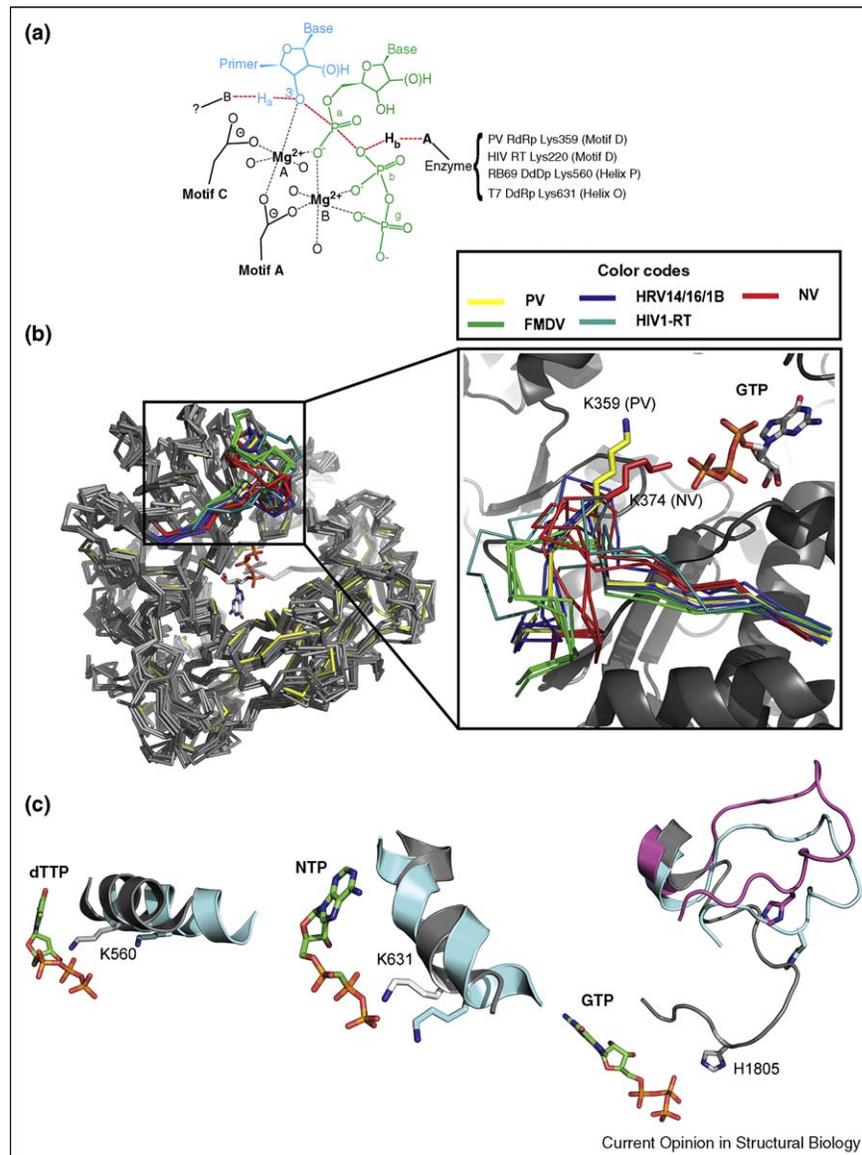
A determinant of nucleotide incorporation efficiency resides on a highly mobile structural element in all nucleic acid polymerases

Nucleic acid polymerases employ a two-metal-ion mechanism for catalysis [15]. One metal (B in Figure 3a) enters the active site with the nucleotide; the other (A in Figure 3a) enters after the nucleotide. Metal B likely influences organization of the triphosphate in the active site. However, metal A lowers the pK_a value for the 3'-OH of the primer and contributes to charge neutralization that forms during the transition state. The acceptor for the proton from the primer 3'-OH is not known. Recent studies of the nucleotidyl transfer reaction catalyzed by the RdRp and other polymerases have made the unexpected observation that two protons are transferred during the reaction, not just one proton [16]. Moreover, it is now clear that the second proton derives from a basic amino acid (general acid) of the polymerase and is transferred to the pyrophosphate leaving group (Figure 3a) [17**]. Protonation of the pyrophosphate leaving group is not absolutely essential but contributes 50-fold to 1000-fold to the rate constant for nucleotide addition. The general acid is located on motif D of RdRps and reverse transcriptases (RTs), on helix O of A-family polymerases (e.g. T7 RNA polymerase), helix P of B-family polymerases (e.g. RB69 DNA polymerase) and the trigger loop of multisubunit RNA polymerases (Figure 3a) [17**].

The general acid is Lys-359 in PV RdRp, Lys-369 in FMDV RdRp, and Lys-220 in the RT from human immunodeficiency virus (HIV). This residue is absolutely conserved in all RdRps and RTs. Motif D is the most dynamic structural element of RdRps and RTs (Figure 3b). Surprisingly, none of the structures available for RdRps or RTs, presumably poised for catalysis, reveal a conformation that is consistent with the ability of this residue to function as a general acid. The general acid (Lys-374) of the RdRp from a Norovirus (NV) comes the closest (Figure 3b).

It is not at all clear why the FMDV RdRp and HIV RT structural studies have not succeeded in capturing the general acid and motif D in its active conformation. Studies of the other polymerases have successfully captured the general acid in the active conformation (Figure 3c). Interestingly, all of the structural elements that contain the general acid undergo substantial changes in conformation after nucleotide binding (Figure 3c). This conformational change may be related to the rate-limiting step (K_2) that is used for the fidelity of nucleotide addition. Consistent with this possibility, amino acid substitutions in helix O of A-family polymerases and the trigger loop of multisubunit RNA polymerases contribute to fidelity [18–22,23*]. If this

Figure 3



Substantial flexibility of a functionally important element observed in all classes of nucleic acid polymerases. **(a)** Extending the two-metal-ion mechanism of nucleotidyl transfer to include general acid catalysis. The positions of general acids of the model polymerases are shown. **(b)** Conserved structural motif D is a dynamic element that approaches the triphosphate moiety of the incoming nucleotide. To the left are shown ribbon representations of the superimposed RdRps from poliovirus (PV), foot-and-mouth disease virus (FMDV), Norwalk virus (NV), and human rhinovirus (HRV) types 14/16/1B, as well as the RT from the human immunodeficiency virus type 1 (HIV1) (amino acid residues 1–315 of the catalytic p66 subunit). The displayed structures were taken from the following PDB files (PV: 1RA6, 1RA7; FMDV: 1U09, 1WNE, 2E9Z; NV: 1SH0, 1SH1, 3BSO; HRV14: 1XR5; HRV16: 1XR7; HRV1B: 1XR6; HIV1-RT: 1RT1, 1RTD). Motif D in the various polymerases is color coded as indicated in the lower right panel. The GTP substrate (from PV binary complex) is shown as sticks at the catalytic cleft. **(c)** Dynamics of structural elements containing the general acid in DNA-dependent polymerases. Comparison of enzyme–primer/template complexes to enzyme–primer/template–nucleotide complexes reveals movement of the general acid in response to nucleotide binding. Movement of helix P (left) in RB69 DdRp created from PDB entries: 1IG9, 2DTU. Movement of helix O in T7 DdRp (center) created from PDB entries: 1S76, 1MSW. Movement of the trigger loop in yeast RNA polymerase II created from PDB entries: 2E2H, 2YU9, 1Y1V. The conformations corresponding to the ternary complexes are gray. The figure is adapted from Ref. [17*].

is also the case for motif D of the RdRp, then this motif may represent an important target to alter fidelity of the viral RdRp for viral attenuation and vaccine development as done for the G64S PV RdRp. Motif D may represent a universal target for the control of viral fidelity and viral

attenuation in all RNA viruses. Also worth noting is the observation that one of the attenuating mutations in the Sabin vaccine strain for PV type 1 converts Thr-362 of the RdRp to Ile [24]. Given its proximity to Lys-359, the Thr-to-Ile substitution at position 362 may impact Lys-359

function. In conclusion, dynamics of motif D will clearly impact function. We suggest that motif D dynamics may hold the key to our understanding of RdRp fidelity and our ability to tune RdRp fidelity for applications such as vaccine development.

Structure of an RdRp precursor cannot explain the absence of polymerase activity

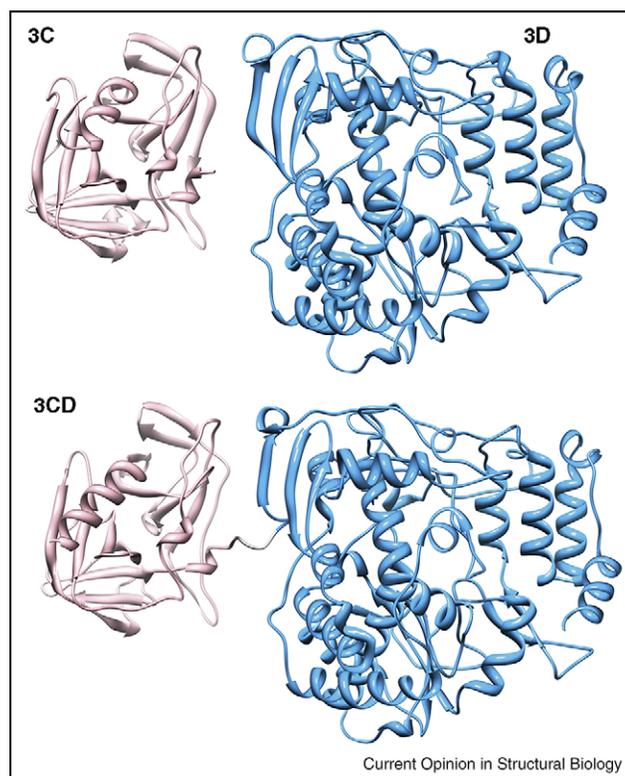
Picornaviruses produce a single polyprotein that is post-translationally processed by viral proteases to produce a variety of incompletely and terminally processed products. For example, in addition to the 3C protease and 3D RdRp, a 3C–3D fusion protein (3CD) is also made. Like 3C, 3CD has protease activity, but unlike 3D, 3CD lacks RdRp activity. The molecular basis for this difference has been of great interest to picornavirologists. A recent suggestion was that the fingers subdomain of 3D was derived from a ‘chameleon’ sequence [25]. As long as the amino terminus of 3D is free to bury itself into the fingers subdomain, then the fingers subdomain would fold into conformation suitable for RdRp function [25]. When the amino terminus of 3D is fused to 3C, however, a new conformation that is inconsistent with RdRp function would be realized. The first structure of a picornaviral 3CD protein was solved recently [26*]. As shown in Figure 4, 3CD is a composite of 3C and 3D. The only hypothesis that could be put forward to explain the absence of RdRp activity in 3CD was that the dynamics of the 3D domain of 3CD may be negatively impacted [26*].

Processing intermediates have the ability to expand the viral proteome, potentially creating new functions or, as described here, inactivating some functions. Moreover, a single amino acid change of a residue that influences protein dynamics can be sufficient to alter the global dynamics of a protein and potentially its function [27]. If this is the case, then the combination of a viral quasispecies and variable extents of polyprotein processing creates unlimited possibilities for the acquisition and evolution of function by a virus with limited coding capacity.

Future directions: exploiting RdRp structures for the discovery of dynamics–function relationships

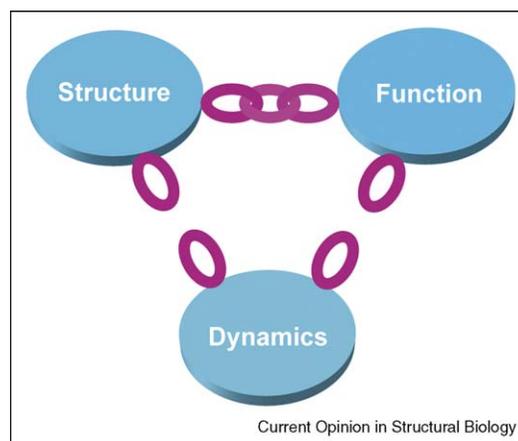
In spite of all of the structural information now available for the RdRp (see Supplementary Table 1), many gaps exist in our understanding of RdRp function. We would like to suggest that dynamics may represent the missing link to our complete understanding of RdRp function (Figure 5). Given the numerous high-resolution structures available for the RdRp in the same and different families, it should be possible to use molecular dynamics (MD) simulations to determine the extent to which dynamics coevolves with structure and function. In addition, the impact of mutations or fusions on dynamics

Figure 4



Structure is unable to reveal the basis for the absence of activity for polymerase precursors. The polymerase precursor 3CD (pdb 2IJD) is a composite of 3C (pdb 1L1N, pink) and 3Dpol (pdb 1RA6, light blue) [26*].

Figure 5



Dynamics: the missing link to a complete understanding of RdRp function.

should be tractable by using MD. Indeed, MD has become extraordinarily powerful [28]. A recent study on HIV RT reported the use of a 360 ns simulation to explain the impact of a non-nucleoside RT inhibitor on HIV RT dynamics [29*]. It should also be possible to

use hydrogen-deuterium exchange mass spectrometry (HDXMS) to evaluate RdRp dynamics. HDXMS should permit analysis of RdRp dynamics in complex with substrates, as well as when fused to other proteins. Again, HDXMS studies of HIV RT have recently been used to identify regions of RT that change conformation in the absence of substrates [30^{*}]. Finally, the molecular weight of most viral RdRps is in the 50–65 kDa range. Enzymes in this size range can now be studied by using nuclear magnetic resonance spectroscopy, which offers an empirical approach to the evaluation of dynamics on the widest range of timescales.

Conclusion

RdRp fidelity may be a tractable target for viral attenuation and vaccine development. Our current understanding of the basis of RdRp fidelity is incomplete even with the availability of multiple crystal structures. Computational and empirical studies of RdRp dynamics may complete our understanding of RdRp function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.sbi.2009.10.012](https://doi.org/10.1016/j.sbi.2009.10.012).

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