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Incorporation fidelity of the viral RNA-dependent RNA polymerase: a kinetic, thermodynamic and structural perspective

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Abstract

Positive-strand RNA viruses exist as a quasi-species due to the incorporation of mutations into the viral genome during replication by the virus-encoded RNA-dependent RNA polymerase (RdRP). Therefore, the RdRP is often described as a low-fidelity enzyme. However, until recently, a complete description of the kinetic, thermodynamic and structural basis for the nucleotide incorporation fidelity of the RdRP has not been available. In this article, we review the following: (i) the steps employed by the RdRP to incorporate a correct nucleotide; (ii) the steps that are employed by the RdRP for nucleotide selection; (iii) the structure-based hypothesis for nucleotide selection; (iv) the impact of sites remote from the active site on polymerase fidelity. Given the recent observation that RNA viruses exist on the threshold of error catastrophe, the studies reviewed herein suggest novel strategies to perturb RdRP fidelity that may lead ultimately to the development of antiviral agents to treat RNA virus infection.

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1. Introduction

Positive-strand RNA viruses cause a number of human diseases, including the common cold, myocarditis, hepatitis and severe acute respiratory syndrome (SARS). Treatment of RNA virus infection has proven to be a challenging task. However, even if effective therapeutics existed, the quasi-species nature of RNA viruses would facilitate the emergence of virus variants that are resistant to antiviral therapy.

The variability displayed in RNA virus populations can be attributed to mutations introduced into the genome by the viral replicase during each round of replication. The viral replication machinery is a complex assembly of viral, and in some cases, host proteins. However, at the heart of the replication machinery is the virus-encoded RNA-dependent RNA polymerase (RdRP). RdRPs have been shown to display a high mutation rate compared to their DNA counterparts (Drake, 1993, 1999). This decrease in fidelity exhibited by RdRPs has been suggested to be the primary contributing factor for the variability in RNA virus genomes.

Numerous studies have documented the high mutation frequency exhibited by RNA viruses as well as their rapid rates of evolution (De la Torre et al., 1990; Domingo, 1989; Domingo et al., 1985, 1996; Drake, 1993, 1999; Drake and Holland, 1999; Holland et al., 1982, 1990). The determination of RNA virus mutation frequencies has been accomplished by evaluating the following: (i) development of resistance to antiviral agents; (ii) escape from monoclonal antibody treatment; (iii) changes in virus fitness; (iv) serial passage of the virus in cell culture or in vivo followed by direct sequencing (Table 1). Unfortunately, mutations that produce replication-incompetent or perhaps translation-incompetent genomes are not scored in these assays. Therefore, direct measurements of the intrinsic error rate of the RdRP obtained from in vitro analysis is the simplest approach to define the upper limit for the mutational frequency of a RNA virus.

The genetic variability of RNA viruses is advantageous and allows viruses to adapt to environmental changes more
rapidly; however, a consequence of this same genetic variability is the enhanced sensitivity of the viral population to accumulation of additional mutations (Holland et al., 1990; Irurzun et al., 1992; Lee et al., 1997). As the number of mutations in the viral genomes increases and passes this error threshold, the fitness decreases significantly, ultimately resulting in extinction. Therefore, agents that increase the mutation frequency of the virus should be effective antivirals. This prediction was confirmed by studies of ribavirin that showed that the mechanism of action of this antiviral agent is lethal mutagenesis (Crotty et al., 2001, 2000).

2. Kinetic mechanism of nucleotide incorporation for poliovirus 3Dpol

In order to establish a system to preclude heterogeneous binding of 3Dpol to primer/template, thereby permitting mechanistic analysis, a novel substrate was designed (Fig. 1) (Arnold and Cameron, 2000). This RNA substrate consists of a 10-nucleotide homopolymeric RNA that is self-complementary and forms a six base-pair duplex region with an identical 4-nucleotide 5′-overhang on both sides of the primer/template. Both 3′-hydroxyls of the substrate are competent for templated extension resulting in a symmetric substrate termed sym/sub (Arnold and Cameron, 2000) (Fig. 1). The enzyme binds productively to sym/sub regardless of the orientation of binding and regardless of which of the two 3′-hydroxyls is in the catalytic center. The 4-nucleotide template permits either single or multiple rounds of nucleotide incorporation to be evaluated by selecting the appropriate nucleotide or nucleotides employed in the reaction. 3Dpol–sym/sub complexes recapitulate a biologically relevant elongation complex (Gohara et al., 2000); therefore, analysis of the mechanism of nucleotide incorporation in vitro using this substrate is likely to be relevant to the reaction in vivo.

Evaluation of the kinetics of nucleotide incorporation into sym/sub by 3Dpol has revealed information on the binding of the incoming nucleotide and the specificity of nucleotide incorporation and the specificity of nucleotide incorporation (Table 2). These parameters are expressed by the kinetic constants: $k_{d,app}$, $k_{pol}$ and $K_{d,app}$, respectively (Arnold and Cameron, 2004b). The fidelity of the viral RdRP can be obtained by comparing $k_{pol}/K_{d,app}$ values for correct and incorrect nucleotides. A complete kinetic and thermodynamic analysis of poliovirus 3Dpol-catalyzed nucleotide incorporation revealed that nucleotide incorporation can be described by the five steps shown in Scheme 1 (Arnold and Cameron, 2004b). The 3Dpol–sym/sub complex (ER) binds nucleotide (NTP) to form a ternary complex (ER$_n$NTP) that under-

| Virus       | Mutation frequency | Assay                        | References                      |
|-------------|--------------------|------------------------------|---------------------------------|
| PV-1        | $5.4 \times 10^{-2}$ | Biochemical assay$^a$         | Ward et al. (1988)              |
| PV-1        | $2.1 \times 10^{-4}$ | GuaD-Gua$^b$                 | De la Torre et al. (1990)       |
| PV-1        | $7.8 \times 10^{-5}$ | GuaD-Gua$^b$                 | Pinero et al. (1995)            |
| Coxsackie A9 | $1 \times 10^{-4}$  | 2-HBB resistance$^c$         | Eggers and Tamari (1965)        |
| Influenza A | $1.5 \times 10^{-5}$ | Plaque assay$^c$              | Purov et al. (1986)             |
| VSV         | $1 \times 10^{-1}$  | Plaque assay$^d$              | Stichlhuber et al. (1989)       |
| PV-1        | $2.1 \times 10^{-4}$ | Sequencing$^e$                | Crotty et al. (2001)            |
| HAV         | $1 \times 10^{-1}$  | Sequencing                   | Sanchez et al. (2003)           |
| TMV         | $3 \times 10^{-4}$  | Sequencing                   | Kramley et al. (1999)           |
| HCV         | $1 \times 10^{-4}$  | Sequencing                   | Young et al. (2003)             |
| FMDV        | $1 \times 10^{-4}$  | Sequencing                   | Ainiosonen et al. (2003)        |
| HTNV        | $1 \times 10^{-3}$  | Sequencing                   | Severson et al. (2003)          |
| LCMV        | $3.6 \times 10^{-4}$ | Sequencing                   | Ruiz-Jaraba et al. (2003)       |

$^a$ Mutation frequency is defined as the number of mutations per replication event.
$^b$ Amount of a noncomplementary nucleotide incorporated divided by the total amount of complementary and noncomplementary nucleotide incorporated using homopolymeric RNA templates.
$^c$ Rescission of guanine dependent to guanine resistant.
$^d$ Development of resistance to 2-(4-hydroxybenzyl)-benzimidazole (2-HBB).
$^e$ Mutations were determined by sequencing different plaques developed after infection with a single clone.
$^f$ Viral population samples from the host organism or cell culture were isolated, amplified and sequenced.

5′GCAUUGGgccct$^a$
3′ccccgggacAg$^c$

Fig. 1. Symmetrical primer template substrate (sym/sub) used to study poliovirus polymerase 3Dpol-catalyzed nucleotide incorporation.
Table 2
Kinetic parameters for 3Dpol-catalyzed nucleotide incorporation

| Substrates          | Kinetic parameters | Fidelity a |
|---------------------|--------------------|------------|
| Nucleic acid        | Nucleotide         | $K_{d,app}$ (nM) | $k_{pol}$ (s$^{-1}$) | $k_{pol}/K_{d,app}$ (nM$^{-1}$ s$^{-1}$) |
| sym/sub-U           | GCA               | 134 ± 18   | 87 ± 4   | 0.65             | –                  |
|                     | CCCUGGGCCC        | 286 ± 00   | 0.80 ± 0.06 | 2.8 × 10$^{-3}$ | 230                |
| 2¢-dATP             | 3¢-dATP           | 317 ± 51   | 1.4 ± 0.1 | 4.4 × 10$^{-2}$ | 150                |
| CTP                 | >500 µM           | <0.0025 x$^{-1}$ | <5 × 10$^{-5}$ | >2.0 x 10$^{5}$ |
| GDP                 | ATTP              | 430 ± 98   | 0.014 ± 0.003 | 3.3 × 10$^{-3}$ | 2.3 x 10$^{3}$ |
| UTP                 | >500 µM           | <0.0025 x$^{-1}$ | <5 × 10$^{-5}$ | >2.0 x 10$^{5}$ |

Fidelity is calculated as $[k_{pol}/K_{d,app}]_{ATP} + [k_{pol}/K_{d,app}]_{incorrect}/[k_{pol}/K_{d,app}]_{incorrect}$ (Patel et al., 1991), reproduced with permission from Biochemistry.

Scheme 1. Complete kinetic mechanism for 3Dpol-catalyzed nucleotide incorporation.

3. Kinetic basis for fidelity of nucleotide incorporation

It has been well documented that substitution of Mn$^{2+}$ for Mg$^{2+}$ as the divalent cation cofactor in polymerase-catalyzed reactions decreases the stringency of substrate selection and incorporation fidelity (Arnold et al., 1999; Beckman et al., 1985; Goodman et al., 1983; Huang et al., 1997; Liu and Tsai, 2001; Tabor and Richardson, 1989). However, the detailed mechanistic basis for the destructive effects of Mn$^{2+}$ was not completely understood until recently (Arnold et al., 2004). By using Mn$^{2+}$ as the divalent cation cofactor, the ability to diminish the rate of phosphoryl transfer for incorrect nucleotides relative to correct nucleotides is lost completely, leaving only the conformational-change step for nucleotide selection (Scheme 2) (Arnold et al., 2004). When Mn$^{2+}$ is employed as the divalent cation, the conformation of the enzyme, and at the same time, organizes the active site for acceptance of the second metal ion required for catalysis (Fig. 2B). Finally, catalysis occurs (Fig. 2C).

Scheme 2. Comparison of the conformational-change step and the phosphoryl-transfer step for 3Dpol-catalyzed correct and incorrect nucleotide incorporation in the presence of Mg$^{2+}$ and Mn$^{2+}$.

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the metal-bound triphosphate coupled with the additional adventitious interactions that can occur between the enzyme and Mn$^{2+}$ increases the stability of the activated ternary complex (Scheme 2, step 2). Compared to reactions in the presence of Mg$^{2+}$, the increase in stability of the activated ternary complex is the same regardless of the nature of the nucleotide (correct or incorrect). In addition, the capacity of Mn$^{2+}$ to bind more tightly to the β and γ phosphates makes the orientation of the triphosphate independent of interactions with residues in the ribose-binding pocket. Consequently, perturbations in the orientation of the triphosphate will not occur in response to binding of a nucleotide with an incorrect base or sugar configuration. Therefore, the inability to couple the nature of the bound nucleotide to the efficiency of phosphoryl transfer is the primary reason for the observed loss of 3Dpol fidelity in the presence of Mn$^{2+}$.

Given that the conformational-change step (step 2) and the phosphoryl-transfer step (step 3) are partially rate-limiting for correct nucleotide incorporation, it was likely that these two steps were used by the enzyme to discriminate a correct nucleotide from an incorrect nucleotide. Through evaluation of the differences between correct and incorrect nucleotide incorporation catalyzed by 3Dpol, it was found that there is no difference in ground-state binding (step 1) regardless of the nucleotide substrate employed (Table 2, Fig. 3). Therefore, ground-state binding cannot contribute to the process of nucleotide substrate selection. The reason for this observation likely reflects the use of the triphosphate for ground-state binding instead of the ribose or base. However, the two steps in the kinetic mechanism for nucleotide incorporation catalyzed by 3Dpol that provide the greatest contribution to fidelity are formation of the activated ternary complex (step 2) and phosphoryl transfer (step 3) (Schemes 1 and 2, Fig. 3).

The conformational change preceding catalysis is thought to be reorientation of the metal-complexed triphosphate moiety of the nucleotide from its ground-state configuration (Fig. 2A) to the catalytically competent configuration (Fig. 2B) (Arnold et al., 2004). The greatest number of favorable interactions will occur with the correct nucleotide, permitting this step to provide some discrimination between correct nucleotides and nucleotides containing an incorrect base or sugar configuration.
base or sugar configuration. The presence of an incorrect sugar or base in the nucleotide-binding pocket can modify the nature and/or strength of the interactions with the actively oriented triphosphate causing a suboptimal organization of the complex for catalysis (Fig. 4).

In addition to the significant destabilization of the activated ternary complex, changes in the phosphoryl-transfer step occur when incorrect nucleotide substrates are utilized (Scheme 2, step 3). The decreased rate of chemistry for incorrect nucleotides is caused by the inability to maintain the triphosphate in the catalytically competent conformation and to maintain the appropriate distance between the α-phosphate and the 3′-OH (Scheme 2, step 3; Fig. 4).

4. Structural basis for fidelity

Sequence alignments of animal virus RdRPs have indicated the presence of several absolutely conserved amino acid residues that can be mapped to the nucleotide-binding pocket (Gohara et al., 2000, 2004; Hansen et al., 1997; Koonin, 1991). Six of these interact with the nucleotide substrate: Asp-233, Asp-238, Asp-328, Ser-288, Thr-293, and Asn-297 (Fig. 5A). In order to determine the importance of these residues for nucleotide selection, 3Dpol derivatives were created in which some of these residues were changed to alanine. The derivatives were subsequently purified and the mechanism of nucleotide selection determined by evaluating the kinetics of incorporation of correct and incorrect nucleotides (Gohara et al., 2000, 2004).

The results obtained from the studies of incorporation of correct and incorrect nucleotides with wild-type 3Dpol and the 3Dpol derivatives permitted the identification of essential amino acid residues and the interactions that are important for correct nucleotide selection. The model for nucleotide binding in Figs. 2 and 4 can be expanded to include these interactions. The first step is binding of the nucleotide in the ground state. In this ground-state configuration, the ribose cannot bind in a productive orientation because the interaction between Asp-238 and Asn-297 observed in the unliganded enzyme occludes the ribose-binding pocket (Gohara et al., 2000, 2004). A conformational change occurs that orients the triphosphate for phosphoryl transfer. This transition is proposed to be partially rate-limiting for correct nucleotide incorporation (step 2 in Scheme 1) (Arnold and Cameron, 2004b; Arnold et al., 2004). Moreover, the stability of the complex in this conformation will dictate the efficiency of phosphoryl transfer as any movement in the position of the triphosphate will produce either a suboptimal orientation or a suboptimal distance for catalysis. In order to maintain the triphosphate in the appropriate orientation, an extensive hydrogen-bonding network is involved that can be traced to residues in the ribose-binding pocket (Fig. 5B) (Gohara et al., 2004). Formation of this network requires reorientation of Asp-238 and Asn-297 as well as interaction of the oxygen of the β-phosphate with the 3′-OH of the nucleotide substrate. The position of the ribose is held firmly by interactions between the 3′-OH and the backbone of Asp-238 and...
by interactions between the 2'-OH and Asn-297. The backbone of Asp-238 is restricted by the interaction of the Asp-238 side chain with other residues in the pocket, perhaps Ser-288 and Thr-293 (Fig. 5B). The appropriate organization of this complex will permit binding and/or alignment of the second divalent cation cofactor, permitting phosphoryl transfer, translocation and pyrophosphate release (Arnold and Cameron, 2004b; Arnold et al., 2004).

Reduced stabilization of the ribose moiety of the bound nucleotide caused by deleting interactions with the 2'-OH (e.g. 2'-dATP or N297A) may also alter the interaction between the 3'-OH and the β-phosphate of the nucleotide substrate, resulting in movement of the triphosphate and the consequent reduction in the efficiency of nucleotide incorporation. This model explains the observation that both the conformational change preceding phosphoryl transfer and phosphoryl transfer are reduced for GMP incorporation by 3Dpol (Arnold and Cameron, 2004b; Arnold et al., 2004) or AMP incorporation by the N297A derivative (Gohara et al., 2000, 2004). Selection against nucleotides with other ribose modifications could employ a similar mechanism. These data are consistent with the conclusion that information on the nature of the interactions in the ribose-binding pocket can be disseminated to the catalytic center by using the conformation of the Asp-238 and the corresponding orientation of the triphosphate. Possible perturbations of the triphosphate caused by binding of a nucleotide with an incorrect sugar or base are illustrated in Fig. 4A and B, respectively. In the G/U mispair, (GMP incorporation into sym/sub-U) the orientation of the triphosphate is altered by the non-planar base-pair. These structural changes explain the observation that both the conformational change preceding phosphoryl transfer and phosphoryl transfer are reduced for GMP incorporation by 3Dpol (Arnold and Cameron, 2004b; Arnold et al., 2004) or AMP incorporation by the D238A derivative (Gohara et al., 2000, 2004). A purine-purine mispair likely disturbs the orientation of Asp-238 in the binding pocket, initiating a cascade of destabilizing events, movement of the 3′-OH, the β-phosphate and ultimately the entire triphosphate moiety of the incorrect nucleotide substrate. A pyrimidine-pyrimidine mispair likely increases the mobility of residues within the pocket, altering the orientation of Asp-238.

The orientation of the triphosphate moiety of the nucleotide substrate is fundamental for nucleotide incorporation not only for the RdRP but also for other polymerases (Beese et al., 1993; Cheetham and Steitz, 1999; Doublie et al., 1998; Johnson et al., 2003; Li et al., 1998; Yin and Steitz, 2002). Stabilization of the triphosphate conformation requires conserved structural motif A (Fig. 6). Stabilization of the triphosphate-metal complex in the active conformation requires a network of hydrogen bonds provided mostly by the backbone of the residues in motif A (Fig. 6). Therefore, any movement of the motif A side chains located in the sugar-binding pocket will be transmitted through the rest of motif A, consequently perturbing the position of both the sugar and triphosphate and reducing the efficiency of phosphoryl transfer as described above for Asp-238 of 3Dpol. The position of residues in motif A can be altered both by either the base or the ribose of the nucleotide (Fig. 6). Similar to Asn-297 (motif B) for 3Dpol, T7 RNA polymerase uses His-784 (motif B) for hydrogen bonding to the 2′-OH of the NTP substrate (Briea and Sousa, 2000). In HIV RT, Phe-160 (motif B) has van der Waals interactions with the 2′-H of the 2′-dNTP substrate (Gutierrez-Rivas et al., 1999). In the presence of a 2′-OH, Phe-160 will cause movement of Tyr-115 (motif A). Likewise, the presence of a motif B residue in DNA polymerases (e.g. T7, BF, KF, Taq and RB69) will cause movement of motif A via the motif A residue located in the sugar-binding pocket (Beese et al., 1993; Cheetham and Steitz, 1999; Doublie et al., 1998; Johnson et al., 2003; Li et al., 1998; Yin and Steitz, 2002).

Succinctly, the nucleotide-binding pocket of all nucleic acid polymerases with a canonical “palm”-based active site is highly conserved. The site can be divided into two parts: a region that has “universal” interactions mediated by conserved structural motif A that organizes the metals and triphosphate for catalysis and one that has “adapted” interactions mediated by conserved structural motif B that dictate whether ribo- or 2′-deoxynucleotides will be utilized. In the classical polymerase, there is a motif A residue located in the sugar-binding pocket capable of interacting with motif B residue(s) involved in sugar selection. This motif A residue in other polymerases could represent the link between the nature of the bound nucleotide (correct vs. incorrect) to the efficiency of phosphoryl transfer as described herein for Asp-238 of 3Dpol (Gohara et al., 2004). Reproduced with permission from Biochemistry, 2004, submitted. © 1998 Am. Chem. Soc.
Table 3

| Enzyme | Nucleotide | Kinetic parameters | Fidelity |
|--------|------------|-------------------|----------|
|        |            | $K_{d,app}$ | $k_{pol}$ (s$^{-1}$) | $k_{pol}/K_{d,app}$ (μM$^{-1}$ s$^{-1}$) |
| WT     | ATP        | 134 ± 18 | 87 ± 4 | 0.65 | – |
|        | RTP        | 386 ± 42 | 0.011 ± 0.003 | 2.8 x 10$^{-5}$ | 2.3 x 10$^4$ |
|        | GTP        | 430 ± 98 | 0.014 ± 0.001 | 3.3 x 10$^{-5}$ | 2.0 x 10$^4$ |
| G64S   | ATP        | 161 ± 10 | 32 ± 2 | 0.20 | – |
|        | RTP        | 367 ± 43 | 0.0021 ± 0.0002 | 5.7 x 10$^{-6}$ | 3.5 x 10$^4$ |
|        | GTP        | 444 ± 66 | 0.0035 ± 0.0002 | 7.9 x 10$^{-6}$ | 2.5 x 10$^4$ |

* Fidelity is calculated as \(\frac{\left(k_{pol}/K_{d,app}\right)_{ATP} + \left(k_{pol}/K_{d,app}\right)_{incorrect}}{\left(k_{pol}/K_{d,app}\right)_{incorrect}}\)

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5. Amino acid residues at remote sites also contribute to RdRP fidelity

Recently, a poliovirus variant with decreased sensitivity to ribavirin was isolated (Pfeiffer and Kirkegaard, 2003). This poliovirus variant encodes a polymerase with a change of Gly-64 to Ser (G64S) mutation in the fingers subdomain (Pfeiffer and Kirkegaard, 2003). Analysis of the mutation frequency of the G64S virus by using a guanidine-resistance assay indicated that the G64S polymerase had an increase in incorporation fidelity.

The fidelity of the G64S 3Dpol has been evaluated by analyzing the incorporation of AMP, GMP and RMP (ribavirin) into sym/sub (Table 3) (Arnold and Cameron, 2004a). Nucleotide binding by G64S 3Dpol is equivalent to wild-type 3Dpol regardless of the nature of the nucleotide, correct or incorrect, consistent with the finding that binding is governed primarily by the triphosphate moiety (Table 3). However, the overall efficiency of RMP and GMP incorporation was reduced significantly relative to wild-type 3Dpol, suggesting a decrease in either the conformational-change step or the phosphoryl-transfer step (Table 3). The fidelity of G64S 3Dpol increased compared to wild-type enzyme, and the capacity of the G64S substitution to permit decreased utilization of RTP could be explained by increased fidelity of this derivative relative to wild-type 3Dpol. The mechanistic basis for this increased fidelity is a change in the stability of the activated ternary complex compared to wild-type 3Dpol (Arnold and Cameron, 2004a). The ability to show that an amino acid substitution in 3Dpol that increases polymerase fidelity causes a change in step 2 (Scheme 1) provides an undisputable link between this step and fidelity (Showalter and Tsai, 2002). The structural basis for the increased fidelity observed for G64S 3Dpol is not currently known and may be difficult to discern, given the subtle difference in fidelity relative to wild-type and the lack of available RdRP co-crystal structures with RNA primer/template and nucleotide bound. The Gly-64 residue is located in the fingers domain (Fig. 7) of the polymerase, and it is not possible to suggest a direct mechanism for interaction with the nucleotide substrate given its remote location.

6. Implications of the kinetic, thermodynamic and structural basis for RdRP fidelity on the development of antivirals

The discovery that residues remote from the ribose-binding pocket can modulate fidelity is quite provocative. It is conceivable that small molecules can be developed that bind to surfaces of the polymerase and modulate fidelity. Compounds that increase incorporation fidelity should make the virus more susceptible to pressures that could be evaded by population diversity, for example, the immune system. Compounds that decrease incorporation fidelity should force the virus into error catastrophe. Clearly, this strategy will prevent the complications associated with nucleoside-based polymerase inhibitors. Importantly, these small molecules can be “recycled” in a mechanism in which the antiviral would bind...
to the activated ternary complex, altering its fidelity and then be released.

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