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Methyl Relaxation Measurements Reveal Patterns of Fast Dynamics in a Viral RNA-directed RNA Polymerase

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Abstract

Molecular dynamics (MD) simulations combined with biochemical studies have suggested the presence of long-range networks of functionally relevant conformational flexibility on the nanosecond timescale in single-subunit RNA polymerases in many RNA viruses. However, experimental verification of these dynamics at a sufficient level of detail has been lacking. Here we describe the fast, picosecond-nanosecond dynamics of an archetypal viral RNA-directed RNA polymerase (RdRp), the 75-kDa P2 protein from cystovirus ϕ 12, using analyses of ^1H - ^1H dipole-dipole cross-correlated relaxation at the methyl positions of Ile (δ 1), Leu, Val and Met residues. Our results, that represent the most detailed experimental characterization of fast dynamics in a viral RdRp till date, reveal a highly connected dynamic network as predicted by MD simulations on related systems. Our results suggest that the entry portals for template RNA and substrate NTPs are relatively disordered, while conserved motifs involved in metal binding, nucleotide selection and catalysis display greater rigidity. Perturbations at the active site through metal binding or functional mutation affect dynamics not only at the immediate vicinity but also at remote regions. Comparison with the limited experimental, and extensive functional and *in silico* results available for homologous systems suggest conservation of the overall pattern of dynamics in viral RdRps.

In RNA viruses, the production of viral proteins and recapitulation of the virus' genome within the host cell constitute two critical events necessary to propagate infection. These are mediated by two processes – transcription, the synthesis of plus-strand RNA to be utilized as mRNA by cellular ribosomes for viral protein translation; replication, the synthesis of minus-strand RNA to recreate the genome in minus- or double-stranded RNA viruses or for use as templates to produce viral mRNA in plus-stranded RNA viruses. Both processes require the activity of a virus-encoded RNA-directed RNA polymerase (RdRp). While viral RdRps use distinct mechanisms to initiate RNA synthesis, either employing a short protein

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Supporting Information: Figure S1-S4, Tables S1-S3 described in the text; Table S4 – list of available chemical shift assignments for Ile (δ 1 only), Leu, Val and Met methyl positions in wild-type ϕ 12 P2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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or nucleic acid primer or not (*de novo*), they share a similar overall fold (Figure 1A) resembling a cupped right hand with “thumb”, “fingers” and “palm” domains¹. In addition, viral RdRps contain conserved motifs (A-E in the palm domain; F in the fingers domain; Figure 1B) that serve to bind RNA template (and primer, where applicable), catalytic and structural divalent metal ions (Mg^{2+}/Mn^{2+}) and to stabilize the nascent daughter chain.

Viral RdRps utilize dynamics on multiple timescales to directly or indirectly modulate function² often with minimal perturbations in the static structures of their catalytic elements. Notably, remote events such as a mutation (G64S) in the poliovirus (PV) RdRp (3Dpol)³ or the binding of an inhibitor on the thumb domain of the hepatitis C virus (HCV) RdRp (NS5B)⁴, both occurring at considerable distances from the active site lead to profound effects on the activities of the respective RdRps without any significant structural changes at the catalytic site. All-atom molecular dynamics (MD) simulations have suggested that the dynamics in both these specific cases show significant alterations with respect to the wild-type, resting enzymes^{5, 6}. Indeed MD simulations on a range of viral RdRps⁵⁻⁸ have provided tremendous insight into conformational dynamics not captured by crystal structures. These simulations have also revealed networks of residues that couple the dynamics of the catalytic site with those in remote regions⁹. However, the experimental determination of the dynamic landscape viral RdRps in as fine detail as is possible is critical to complement the *in silico*, and the biochemical/functional data available for a wide range of viral RdRps. This understanding may help in the design of unique anti-viral strategies that target the dynamics of remote sites that are dynamically coupled to the catalytic machinery¹⁰ perhaps through the generation of mutator strains with increased fidelity reducing the relative population of drug-resistant variants thus making the species more susceptible to targeted small molecule intervention³.

While solution NMR seems to be the obvious choice for detailed analyses of viral RdRp dynamics, this has been extremely challenging till date largely due to the large sizes of the viral RdRps (typically > 50 kDa) and instability at the relatively high concentrations required for these studies. Nevertheless, NMR studies of viral RdRps are beginning to appear in the literature, including those that suggest dynamics on the catalytic μ s-ms timescale^{11, 12}. However, these studies have relied on a very small number of probes of dynamics e.g. 25 Ile $\delta 1$ positions in the RdRp (P2) from the cystovirus $\phi 6$ ^{11, 12} or 17 Met ϵ positions in PV 3Dpol¹³⁻¹⁵. As described recently¹⁶, we have obtained resonance assignments for a significantly larger number of methyl ^{13}C , 1H resonances in the 75 kDa RdRp (P2) from cystovirus $\phi 12$ enhancing our ability to study the dynamics of a viral RdRp in a far greater level of detail than was previously possible. For $\phi 12$ P2, resonance assignments are now available for 31/32 Ile $\delta 1$ positions, 36/47 Leu δ positions, 37/44 Val γ positions and 18/19 Met ϵ positions providing about 180 individual probes of structure and dynamics (Figure 1C). Using these resonance assignments and measurement of the intra-methyl 1H - 1H dipole-dipole cross-correlation rate (η) that reports on the degree of order of the methyl-rotation (C_3) axis¹⁷, we investigate the structural dynamics of $\phi 12$ P2 (referred to as P2 from hereon forward) on the fast, picosecond-nanosecond timescale. The significantly larger number of probes that are uniformly distributed over *all* the structural domains of P2 and cover *all* its sequence motifs, provide the ability to analyze dynamics in a typical viral RdRp with far better resolution than previously possible. A detailed analysis of these

dynamics and its alteration upon binding divalent metals ions required for catalysis and substrate NTPs or in the presence of a functional mutation¹⁸ suggests a network where remote regions are dynamically coupled to the active site. Further, a re-analysis of the fast dynamics for the 25 Ile $\delta 1$ positions in the homologous $\phi 6$ P2 suggests that the overall patterns of dynamics are conserved in cystoviral RdRps and perhaps more widely.

Materials and Methods

Sample Preparation

The cloning, expression and purification of $\phi 12$ P2 has been described in detail previously¹⁶ and will not be reproduced here. The *ts*-mutant (T425I)¹⁸ was expressed and purified in identical fashion as wild-type P2. In order to obtain spectra of the highest quality possible for the cross-correlated relaxation measurements (described below) on $\phi 12$ P2 (and on the *ts*-mutant), two separate samples, termed IM and LV, were used. The IM samples were labeled as follows: Ile (¹³CH₃, $\delta 1$ only), Met (¹³CH₃, the Met side-chain was fully protonated), uniformly ²H-labeled; LV samples were labeled as follows: Leu (¹³CH₃, ¹²CD₃), Val (¹³CH₃, ¹²CD₃), uniformly ²H-labeled. P2 samples (~230 μ M) were dissolved in a 100% D₂O-based buffer containing 20 mM Tris at pH 8, 150 mM NaCl and 1 mM DTT. Additional experiments were performed on wild-type $\phi 12$ P2 in the presence of 5 mM MgCl₂ with and without 3 mM GMPCPP (Jena Bioscience GmbH).

Estimation of the Rotational Correlation Time and S_{axis}^2

The rotational diffusion tensor for $\phi 12$ P2 was calculated using the bead model as implemented in the HYDRONMR package¹⁹. The atomic radius, temperature and viscosity were set to 3.2 Å, 298.15 K and 1.089 cP, respectively. High-resolution crystal structures of P2 (PDB: 4IEG, 4GZK)²⁰ were utilized for the calculations. The radius of the solvent shell was automatically determined by the program.

The correlation time, τ_C , of wild-type P2 and the *ts*-mutant (also in the presence of Mg²⁺) were estimated using the 1-dimensional ¹⁵N, ¹H TRACT experiment²¹ (at 600 MHz) that monitors the difference in the relaxation rates of the α -state (R_α) and the β -state (R_β) of the amide ¹⁵N using relaxation delays of 0.11, 1.1, 3.23, 6.45, 9.68, 12.9, 19.35, 25.81 and 34.41 ms. The region between 8 and 10 ppm of the 1-dimensional spectra was integrated and the integrated intensity as a function of the relaxation delay was fitted to a single exponential decay separately for the α - and β -state spectra to obtain R_α and R_β values. The value of τ_C was obtained using a one-dimensional grid-search and the following equations:

$$R_\beta - R_\alpha = \frac{1}{6} \left(\frac{\mu_0}{4\pi} \right) \frac{\hbar^2 \gamma_N^2 \gamma_H B_0 \Delta \delta_N}{r_{NH}^3} (3 \cos^2 \theta - 1) [4J(0) + 3J(\omega_N)]$$

$$J(\omega) = \frac{2}{5} \frac{\tau_C}{1 + \omega^2 \tau_C^2} \quad (1)$$

$r_{NH} = 1.02$ Å, $\delta_N = 160$ ppm, $\theta = 17^\circ$ and all other symbols have their usual meaning. The value of τ_C obtained in 10% D₂O was scaled to that in 100% D₂O using the linear dependence of the correlation time on viscosity in the Stokes-Einstein equation.

Measurement of Intra-methyl Dipole-dipole Cross-correlation Rates

Experiments to determine the intra-methyl ^1H - ^1H dipole-dipole cross-correlation rates¹⁷ were performed at 25°C with separate datasets being acquired for the “allowed” (64 scans per transient) and “forbidden” (64 scans per transient, except for $T = 1$ and 2 ms where 128 scans were used; see below) spectra. Measurements were performed on a 600 MHz Varian Inova spectrometer equipped with a triple-resonance cryogenic probe capable of applying pulsed field gradients along the z-axis. Experiments used sweep-widths of 12 ppm (512 complex points) and 14 ppm (48 complex points) for the ^1H and ^{13}C dimensions, respectively for the IM samples. For the LV sample, the corresponding values were 12 ppm (512 complex points) for the ^1H dimension and 11 ppm (48 complex points) for the ^{13}C dimension. Nine different values of the relaxation delay $T = 1, 2, 4, 6, 12, 16, 18, 22$ and 28 ms were used for the wild-type enzyme in absence or presence of Mg^{2+} and $\text{Mg}^{2+}/\text{GMPCPP}$. For the *ts*-mutant, $T = 1, 2, 4, 6, 10, 14, 18, 22$ and 26 ms, were used. All relaxation data were processed using NMRpipe²² and analyzed using the NMRViewJ²³ software. The intra-methyl ^1H - ^1H dipole-dipole cross-correlation rates (η) were obtained by fitting the experimental data to Equation (2) using in-house software that utilized the ODRPACK library²⁴.

$$\frac{I_f}{I_a} = \frac{-0.5\eta \tanh\left(\sqrt{\eta^2 + \delta^2}T\right)}{\sqrt{\eta^2 + \delta^2} - \delta \tanh\left(\sqrt{\eta^2 + \delta^2}T\right)} \quad (2)$$

Where I_a and I_f are intensities of corresponding peaks in the “allowed” and “forbidden” spectra and δ is a leakage term due to ^1H - ^1H cross-relaxation¹⁷. I_f values were appropriately scaled where necessary to account for the differences in the number of scans used in the “allowed” and “forbidden” spectra. The estimated cross-correlation rates were converted into the generalized order-parameter (S_{axis}^2) using:

$$S_{\text{axis}}^2 = \frac{40}{9} \frac{r_{\text{HH}}^6}{\gamma_H^4 \hbar^2 \tau_C} \eta \quad (3)$$

Where $r_{\text{HH}} = 1.813 \text{ \AA}$ is the intra-methyl ^1H - ^1H distance. All other terms have their usual meaning.

Chemical Shift Perturbations

Chemical shift perturbations were calculated using the following equation:

$$\Delta\delta = \sqrt{\left[\delta_{H,\text{ref}} - \delta_{H,i}\right]^2 + \left[\frac{1}{3.94}(\delta_{C,\text{ref}} - \delta_{C,i})\right]^2} \quad (4)$$

Where $\delta_{H,ref}$ ($\delta_{C,ref}$) and $\delta_{H,i}$ ($\delta_{C,i}$) are the methyl 1H (^{13}C) chemical shifts in the reference state and in the liganded/mutated state, respectively. The 3.94 factor represents the ratio of standard deviations of 1H and ^{13}C chemical shifts averaged over the Ile, Leu, Val and Met residues in the Biological Magnetic Resonance Data Bank (www.bmrb.wisc.edu). The unliganded, apo protein was chosen as the reference state for determining the perturbations induced by the addition of Mg^{2+} ions or by mutations. The perturbations induced by Mg^{2+} /GMPCPP were calculated separately using either the apo or the Mg^{2+} -bound state as reference.

Network Analysis

A T425I mutation was generated *in silico* using the “swapaa” command in UCSF Chimera²⁵ and the structures of $\phi 12$ P2 (PDB: 4IEG, 4GZK)²⁰. Protons were added to the structures of both the wild-type and the *in-silico* generated *ts*-mutant that were subsequently minimized using the AMBERff99SB force-field and standard protocols implemented in UCSF Chimera²⁵. Network analysis was then performed on the minimized structures using the RINalyzer^{26, 27} plug-in for the Cytoscape (version 3.1.1) platform. Residue Interaction Networks (RINs) were generated from the static structures considering non-covalent interactions utilizing both backbone and sidechain positions using default parameters. The shortest path closeness values for all nodes (residues) to residue 425 were computed using the number of interactions as the edge weight; multiple edges were handled using sum of weights; scores were converted into distances using the |max-value| criterion; and the cutoff for the weighted degree was set to 5. Shortest path closeness scores for Ile, Leu, Val and Met residues were subsequently renormalized to obtain Π values using the following equation

$$\Pi = \frac{C_i}{C_{ILVM,max}} \quad (5)$$

Where C_i is the closeness score for a particular Ile, Leu, Val or Met residue; $C_{ILVM,max}$ is the maximum closeness score obtained for an Ile, Leu, Val or Met residue. Thus $\Pi=1$ implies that a particular residue has the highest closeness score among all Ile, Leu, Val and Met residues in the structure.

Results and Discussion

Measurement of Fast Dynamics of Methyl Groups

Given that for large proteins such as P2 conventional T_1 , T_2 and $\{^1H\}$ - ^{15}N NOE based approaches to determine the overall hydrodynamic properties²⁸ become inefficient, we pursued alternative strategies. Calculations using the available structures of P2²⁰ and the bead model as implemented in the HYDRONMR package¹⁹ suggests a small degree of anisotropy in the rotational diffusion tensor ($D_{para}/D_{perp}=1.17$). We did not expect that the neglect of this rotational anisotropy would lead to significant errors in our analyses. Therefore, assuming isotropic tumbling, the overall rotational correlation time (τ_C) for apo P2 was obtained from a 1D ^{15}N , 1H TRACT experiment²¹ measured in a buffer prepared in 90% H_2O and 10% D_2O . The difference in decay rates of the amide α -state (R_α) and β -state

(R_B) envelopes yields a value of $67.8 \pm 2.8 \text{ sec}^{-1}$ corresponding to a τ_C value of $33.0 \pm 1.4 \text{ ns}$. Using a scaling factor of 1.23 to account for the change in solvent viscosity in going from a $\sim 10\%$ to $\sim 100\%$ D_2O -based buffer leads to a τ_C value of $40.6 \pm 1.7 \text{ ns}$. Similar values (within error) were obtained for additional states of wild-type P2 and for the *ts*-mutant. Thus a τ_C value of 41 ns was used in all calculations.

In order to measure dynamics of the methyl rotation-axis (C_3 -axis) on the ps-ns timescale, we measured the intra-methyl 1H - 1H dipole-dipole cross-correlation rate (η) (Figure 2) using the pulse sequences developed by Tugarinov *et. al*¹⁷. The S_{axis}^2 values were estimated from the η and τ_C determined above using Equation 3. A total of 134 S_{axis}^2 values for the wild-type enzyme in the absence of ligands could be measured with a high degree of accuracy (Table 1). Resonances with significant spectral overlap and those with η values (average $94.8 \pm 42.6 \text{ s}^{-1}$; 25% trimmed mean: $101.3 \pm 41.2 \text{ s}^{-1}$) with errors larger than 25 s^{-1} were excluded from detailed analysis. The S_{axis}^2 values in apo P2 show relatively weak correlation with simple structural features such as solvent exposure, local packing density etc., as noted in previous studies²⁹. The following overall trend is seen for P2 in the apo state: $S_{axis}^2(\text{Val}, \gamma 1/2) \sim S_{axis}^2(\text{Ile}, \delta 1) > S_{axis}^2(\text{Leu}, \delta 1/2) > S_{axis}^2(\text{Met}, \epsilon)$ (Table 1). This overall trend is maintained for the various liganded states and in the *ts*-mutant discussed below.

Influence of Divalent Metal Ions on Fast Dynamics

RNA and DNA polymerases utilize divalent metal ions (Mg^{2+}) to catalyze nucleic acid polymerization³⁰. MD simulations on HCV NS5B have indicated that binding of Mg^{2+} produces significant alterations in RdRp dynamics⁷ notably at regions distant from the binding site. In order to test this scenario for P2, η values were measured in the presence of saturating amounts of Mg^{2+} . Presence of metal ions did not elicit large-scale changes to the average structure of P2 as reflected by the absence of large chemical shift perturbations in the assigned methyl resonances. Based on the perturbations, the largest structural effects were found to localize to the palm domain in the conserved motifs near the metal binding sites (Note: Mg^{2+} ions occupy only non-catalytic structural sites that are displaced by around 8 Å from the canonical catalytic sites²⁰ in the absence of NTPs). Residues that display significant shift perturbations (calculated using Equation 4) include V350 (motif A, 0.18 ppm), I465 (motif C, 0.06 ppm), L483 (0.08 ppm) and L488 (motif D, 0.07 ppm). Some residues distant from the metal-binding site also show perturbations; the largest of these is seen for M48 (0.07 ppm).

Substantial changes in dynamics as reflected by alterations in the S_{axis}^2 values compared to apo P2 (ΔS_{axis}^2 is defined as $S_{axis}^2(\text{apo}) - S_{axis}^2(Mg^{2+})$, thus negative values imply greater order in the presence of Mg^{2+} ions) found in the palm domain proximal to the metal binding site (Table S1). V434 (-0.36 ± 0.23) and I465 (-0.22 ± 0.15) belonging motif B and motif C, respectively, display the largest overall changes in S_{axis}^2 values in the presence of Mg^{2+} . Significant ΔS_{axis}^2 values are also seen at remote sites (Figure 3, identified as those having no heavy atoms within 15 Å of any heavy atom at the binding site) at the top of the fingers

domain near the template entry channel (e.g. L402, $\Delta S_{\text{axis}}^2 = -0.14 \pm 0.11$), bottom of the fingers domain (e.g. I168, $\Delta S_{\text{axis}}^2 = -0.23 \pm 0.10$) and in the C-terminal domain (I629, $\Delta S_{\text{axis}}^2 = -0.22 \pm 0.13$). The experimental data supports long-range coupling to the catalytic region as predicted originally by the MD simulations of Davis and Thorpe⁷ on HCV NS5B and by Moustafa *et al.*⁶ on PV 3Dpol. Interestingly, most of the statistically significant changes in S_{axis}^2 values in the presence of Mg^{2+} are less than zero (Table S1, Figure 3) suggesting an overall decrease in flexibility. However, two of the only three residues that display significant $\Delta S_{\text{axis}}^2 > 0$ (V473 on motif C and M497 on motif D, Table S1) belong to the conserved motifs that form the catalytic site. Others residues located on the conserved motifs e.g. V434 (motif B) and I465 (motif C) showed significant $\Delta S_{\text{axis}}^2 < 0$ (Table S1). This could indicate a redistribution of conformational entropy within the catalytic cavity upon metal binding.

Influence of the Binding of GTP Analogs

GTP has been considered to be the initiation nucleotide in several *de novo* initiating viral RdRps including those from bovine viral diarrhea virus (BVDV), HCV, broom mosaic virus (BMV) and indeed, cystoviruses³¹. The reason for this could be that since these viral genomes contain pyrimidine-rich 3'-ends and a purine nucleotide is required to form the correct initiation complex. However, it has been suggested that GTP could bind to the RdRp prior to transcriptional initiation acting as a mimic of the product RNA³². As in the case of Mg^{2+} ions above, we investigated whether $\text{Mg}^{2+}/\text{GTP}$ (the non-hydrolysable analog GMPPCP was used) could also perturb the dynamics distant from the active site. The addition of $\text{Mg}^{2+}/\text{GMPCPP}$ resulted in no significant chemical shift perturbations compared with Mg^{2+} alone and none of the resonances display δ values > 0.03 ppm suggesting limited effects on the mean structure. However, significant changes in S_{axis}^2 values relative to the Mg^{2+} -bound state were seen ($\Delta S_{\text{axis}}^2 = S_{\text{axis}}^2(\text{Mg}^{2+}) - S_{\text{axis}}^2(\text{Mg}^{2+}/\text{GMPCPP})$; Table S1). Several of these are located at positions remote from the catalytic site in fact I168 located on the bottom of the fingers domain showed one of the highest ΔS_{axis}^2 values (0.20 ± 0.14) (Figure 3; Note: The positions of the GTP molecules in Figure 3 are modeled on their corresponding positions in the ternary complex of $\phi 6$ P2 with a short DNA template and 2 GTPs (PDB: 1HI0)³³; it has been noted that the occupancy of NTP sites is between 1 and 2 in the absence of primer and the NTP molecules are slightly displaced from their positions in a ternary complex³⁴). Interestingly, most of the changes imply increased flexibility ($\Delta S_{\text{axis}}^2 > 0$ in most cases) in the $\text{Mg}^{2+}/\text{GMPCPP}$ state compared to Mg^{2+} -alone. In fact overall, there are few statistically significant ΔS_{axis}^2 values, largely localized in the vicinity of the binding sites (Figure S1), when comparing the apo and the $\text{Mg}^{2+}/\text{GMPCPP}$ states. This seems to indicate that GMPCPP binding largely reverses the increased rigidity seen in P2 in the presence of Mg^{2+} alone especially at remote locations distant from the catalytic site. That this effect is not an artifact of the removal of Mg^{2+} ions from P2 by GMPCPP is confirmed by the fact that the P2 resonances do not shiftback towards their corresponding positions in the apo enzyme.

Long-range Conformational Coupling in a Functional Mutant

In order to further assess long-range dynamic coupling in P2 we analyzed a point mutant (T425I, on motif B). This mutant is referred to as a temperature-sensitive (*ts*-mutant) and has a substantially different activity profile compared to wild-type enzyme¹⁸. Wild-type P2 displays maximal polymerase activity at around 30° C while the *ts*-mutant is most active around 10° C and shows an almost linear decrease in activity at higher temperatures becoming almost inactive at 30° C. As seen in Figure 4A, the largest chemical shift perturbations resulting from the mutation are localized near the mutation site though a few residues distant from it also display significant differences in chemical shift between the wild-type and *ts*-mutant proteins. These include M378 (0.06 ppm) and V434 (0.04 ppm) neither of which contain any atom within 10 Å of the mutation site. An analysis of the renormalized shortest path closeness scores (Π , see MATERIALS AND METHODS above) using both wild-type P2 and the computationally generated T425I mutant largely indicates that the residues with significant chemical shift differences ($\delta > 0.04$ ppm) between wild-type and mutant P2 proteins also display significant Π values (> 0.5 , the top left quadrant in Figure 4B). For example the $\delta 1$ position of I362 that has the largest difference in chemical shifts ($\delta = 0.12$ ppm) between wild-type P2 and its *ts*-mutant displays Π values of 0.58 and 0.61 for the wild-type P2 and its *ts*-mutant, respectively. The case of M378 ($\Pi = 0.66$ for wild-type and 0.55 for the *ts*-mutant; $\delta = 0.06$ ppm) discussed above is quite unique in that it is relatively well networked to residue 425 in spite of not being in its immediate vicinity. However not all residues with significant Π values also show very large δ values, this is especially true for the minimized wild-type structure (lower right quadrant of Figure 4B). A standout example is L427 that in spite of having a $\Pi = 1$ (for both wild-type and the *ts*-mutant) has a relatively modest $\delta \sim 0.03$ ppm (Figure 4B). The overall correlation of Π and δ values is modest (Pearson correlation co-efficient of 0.61 for wild-type and 0.65 for the *ts*-mutant). However one cannot expect a perfect correlation especially for side-chain positions between crystallographic conformations for structures solved under cryogenic conditions with NMR data collected at room temperature. The Π and ΔS_{axis}^2 values (discussed below), not unexpectedly, are largely uncorrelated (correlation co-efficient = 0.17 for wild-type and 0.24 for the *ts*-mutant; data not shown).

Compared to the chemical shift changes, long-range effects of the T425I mutation are more pronounced in the dynamics changes between the *ts*-mutant and wild-type P2 as reflected by alterations in S_{axis}^2 values. Based on the crystal structure of P2, we had predicted that T425I mutation would lead to a loss of a hydrogen-bonding interaction leading to increased local mobility²⁰. Indeed I425 in the mutant is highly disordered ($S_{\text{axis}}^2 = 0.26 \pm 0.01$). However, the overall changes in dynamics suggest increased rigidity (as reflected the fact that most $\Delta S_{\text{axis}}^2 = S_{\text{axis}}^2(\text{apo}) - S_{\text{axis}}^2(\text{ts}) < 0$, Table S1) compared to the wild-type enzyme. A residue-by-residue comparison with S_{axis}^2 values in wild-type P2, reveals the expected long-range effects. Several significant ΔS_{axis}^2 values are seen for the remote residues L402 (-0.19 ± 0.14) and L222 (-0.19 ± 0.16) both from the fingers domain (Figure 3). However, the most substantial ΔS_{axis}^2 value is seen for I629 (-0.25 ± 0.21) that lies on the C-terminal domain that has no atoms within 19 Å of the site of mutation.

Anisotropic Motions

In addition, to analyzing S_{axis}^2 values for P2 in the apo, Mg^{2+} or $\text{Mg}^{2+}/\text{GMPCPP}$ -bound states and those for the *ts*-mutant, we specifically compared S_{axis}^2 values for resolved $\gamma 1/\gamma 2$ and $\delta 1/\delta 2$ positions in Val and Leu residues, respectively. In apo P2, several residues display significant differences in the S_{axis}^2 values between their prochiral methyl positions (Table S2). In the case of L483 ($|\Delta S_{\text{axis}}^2| = 0.09 \pm 0.03$, Table S2) the S_{axis}^2 values are quite low ($S_{\text{axis}}^2 = 0.27 \pm 0.02, 0.18 \pm 0.01$); the ^{13}C chemical shift difference between the prochiral positions is also small (0.64 ppm) suggesting roughly equal populations³⁵ of the *trans* and *gauche*+ configurations and extensive averaging about the $\chi 2$ dihedral angle. However, given the asymmetry in the S_{axis}^2 values there appears to be additional averaging on the fast timescale independent of the averaging about $\chi 2$. This motional axis has different projections onto the two methyl C_3 axes leading to differential effects. Anisotropic motions could explain the asymmetric S_{axis}^2 values for L427 ($|\Delta S_{\text{axis}}^2| = 0.06 \pm 0.11$, Table S2) though there is no significant $\chi 2$ averaging in the fast (or perhaps in any) timescale and this is reflected in the high S_{axis}^2 ($0.91 \pm 0.05, 0.75 \pm 0.06$) and the significant ^{13}C chemical shift difference (3.01 ppm) between the two prochiral positions, with the latter suggesting a ~80:20 partitioning between the two rotameric states.

The number of residues that display significant anisotropy in the S_{axis}^2 values increases to a large extent in the presence of Mg^{2+} ions. These residues include several that are remote from the Mg^{2+} -binding site e.g. V26 ($|\Delta S_{\text{axis}}^2| = 0.16 \pm 0.02$) and V569 ($|\Delta S_{\text{axis}}^2| = 0.32 \pm 0.07$). The number of residues displaying anisotropic S_{axis}^2 values decreases substantially in the presence of GMPCPP. Thus, Mg^{2+} ions appear not only to induce substantial changes in the overall flexibility but also in the motional anisotropy as reported by the Ile, Leu, Val and Met methyl positions of P2. While the precise details are different, profound motional effects of bivalent cations on the dynamics of viral RdRps has been noted in the MD simulations of Davis and Thorpe⁷ on HCV NS5B.

Overall Flexibility on the Fast Timescale

Another aspect borne out the MD simulations on several homologous RdRps⁵ are the differences in flexibility of individual motifs (A-F) conserved in RdRps. The presence of assigned methyl probes on every sequence motif (Table 2) allows an experimental assessment of these predictions in the case of P2. Unlike in the case of the backbone order parameters, obtaining averages over structural elements or directly comparing S_{axis}^2 values for different residue types may lead to misleading results. This is due to the fact that different atomic positions for the different residue types may have inherent differences in their flexibility based on distance from the backbone and on local geometry²⁹. For example, on average, the S_{axis}^2 values for the Met C ϵ position that measures the flexibility of the S-C ϵ bond tend to be amongst the lowest in proteins, while the S_{axis}^2 values for the Ala C β position, a measure of the flexibility of the C α -C β bond tend, to be the highest²⁹. In order to overcome this problem we defined a parameter Σ that provides a measure of the relative

mobility of a given position independent of residue type. Σ may be calculated from the measured η values using

$$\Sigma = \frac{1}{2} \left[1 + \frac{\eta - \overline{\eta_{\text{type}}}}{|\eta - \overline{\eta_{\text{type}}}|_{\text{max}}} \right] \quad (6)$$

where $\overline{\eta_{\text{type}}}$ is the η value for a particular residue type and $|\eta - \overline{\eta_{\text{type}}}|_{\text{max}}$ is the maximum deviation of a η value from the corresponding average value for that residue type (i.e. Ile, Leu, Val, Met). Thus, $\Sigma = 0$ or $\Sigma = 1$ if a particular residue has the lowest or the highest η value, respectively, for a particular residue type. $\Sigma = 0.5$ if $\eta = \overline{\eta_{\text{type}}}$. Thus Σ values < 0.5 suggest above average mobility. Note that even though Σ is defined for each measured η value, it is a non-local quantity (unlike S_{axis}^2 that is a local quantity) and represents the *relative* mobility of a particular methyl rotation axis compared to all others for which experimental data is available. While it is independent of global effects e.g. changes in rotational correlation time when changing buffers etc., it is not appropriate for use in comparing flexibilities on a residue-by-residue basis. For such cases, local quantities such as S_{axis}^2 (as described above) or η values themselves provide more robust measures. Based on the fact that ranges of S_{axis}^2 values seen in the present case are representative of the dynamic ranges expected in proteins, we do not suspect bias towards a particular dynamic range. Therefore we expect that Σ values obtained here accurately represent the relative mobility even though we do not have assignments (or otherwise could not analyze the data with sufficient level of precision) for all methyl positions for the residue types (Ile, Leu, Val, Met) considered here.

Analysis of Σ values for the well-sampled fingers, thumb and palm domains suggested similar distributions overall (Figure S2) with average values largely around 0.5 (Table S3; the C-terminal domain in the apo state of wild-type P2 is an exception but this could be due to a limited number of probes), suggesting, at least for the methyl-bearing side-chains analyzed here, the domains show similar fast dynamics on average. However, the lowest Σ values (< 0.25) were largely clustered around the mouth of the template entry tunnel and at the entry channel for the substrate NTPs (Figure 5A, left panel) and no positions with Σ values > 0.75 are seen near either entry portal (Figure 5B, left panel). Though a limited number of probes are available for $\phi 6$ P2 (22/25 and 23/25 Ile 61 could be analyzed for the apo and Mg^{2+} -bound states, respectively), the overall distribution of dynamic and rigid methyl groups followed similar patterns (Figures 5A and 5B, right panels) suggesting that the overall pattern of fast dynamics is largely conserved at least in the cystoviral P2 proteins.

A closer inspection of the Σ values of the conserved RdRp structural motifs in P2 indicates that the motifs A, B and to a large extent, C are well ordered while motifs D (its N-terminus is highly disordered while its C-terminus has a higher degree of order), E and F are more disordered, largely in line with the limited data available for $\phi 6$ P2 (Table 2). The spatial distribution of these residues indicates a region of order sandwiched by two regions of disorder (Figure 5C). MD simulations on several RdRps from picornaviruses suggest a

substantial degree of variability in the dynamics of individual motifs, however D, E and F tend to be mobile in most⁵ as is the case here.

Of special interest is the large degree of disorder in motif F for all states considered for $\phi 12$ P2 (there are no probes available for motif F in $\phi 6$ P2). A recent MD analysis of PV 3Dpol backed by functional studies indicates that the active site oscillates between a conformation that is incapable of binding nucleotide and another that is able to bind nucleotide. This motion that involves motif F in 3Dpol purportedly contributes to the first step in nucleotide binding and takes place on the nanosecond timescale³⁶. This motion then facilitates the closing of motif D that comprises the so-called fidelity checkpoint for the incorporation of the correct nucleotide occurring on a slower, millisecond timescale¹⁴. The nanosecond dynamics in motif F is altered in high- (G64S) and low-fidelity (H273R) mutators in 3Dpol. In our studies the motif F residue V299 (that maps onto residue I176 in 3Dpol that been shown to participate in the NTP-binding occluded to NTP-binding competent transition³⁶) shows significant alterations in dynamics upon the addition of Mg^{2+} (Table S1). Further changes are seen for V299 in the presence of GMPCPP ($\Delta S_{axis}^2 = 0.08 \pm 0.06$; but this change did not meet our threshold for statistical significance and is therefore not included in Table S1). Additionally, V299 in the *ts*-mutant is more rigid ($S_{axis}^2 = 0.52 \pm 0.02$) compared to wild-type (0.41 ± 0.03). The relationship to error incorporation in the two-cases is not yet known, though the *ts*-mutant does show slight differences in the relative ease of nucleotide utilization during transcription compared to wild-type enzyme¹⁸.

Conclusions

Our studies on the $\phi 12$ RdRp suggest an enzyme that displays significant dynamic heterogeneity confirming results of MD simulations on homologous systems^{5, 7}. The entry portals for ssRNA templates and substrate NTPs are found to be dynamic. Some catalytic motifs are found to be mostly dynamic (D, E and F) while others are largely rigid (A, B and C). The former are involved template/NTP binding whereas the latter are involved in metal ion binding (A, C) and in nucleotide selection (B). Cystoviral RdRps utilize single-stranded RNA templates of different sequences (albeit with different efficiencies) and various NTPs in their enzymatic reactions. A relationship between dynamics and specificity has been demonstrated before in the immunological maturation of an antibody for the T cell receptor (TCR) where higher specificity is accompanied by a reduction in the range and amplitude of fast dynamics^{37, 38}. It is conceivable that flexibility on the fast timescale allows the enzyme to fine-tune its interactions with templates/substrates with slightly different structural features and in doing so also helps in the efficient redistribution of conformational entropy upon binding³⁹.

Additionally, remote regions appear to be dynamically coupled to the active site on the fast timescale and perturbations at the active site through metal ion binding or point mutations lead to altered dynamics at remote regions. Indeed several of these remote couplings cannot be fully explained by the topological analyses of static crystal structures. As mentioned earlier, MD simulations on 3Dpol have suggested long-range pathways of correlated fast dynamics^{5, 9}. While such detailed *in silico* results are not yet available for P2, a structure-

based mapping of the 3Dpol dynamics onto P2 suggests that many of the changes in dynamics at sites remote from the region perturbed (e.g. by Mg^{2+} binding) would indeed lie on the proposed pathways (Figure S3).

While the dynamics studied here are too fast to have any direct influence on catalysis, they are likely to play a major role in determining the affinity and specificity of template or substrate binding through the influence of conformational entropy⁴⁰. It is also possible that these motions play an indirect role in catalysis through their hierarchical influence on slower, catalytic timescales⁴¹. Studies on 3Dpol have suggested this hierarchical connectivity in dynamic timescales as contributing to the fidelity checkpoint in which fast dynamics in motif F (discussed above) enables the slow closure of motif D upon nucleotide binding. While we have not completed the analysis of slow microsecond-millisecond dynamics in P2, we do find evidence of slow dynamics in motif D as probed by multiple-quantum relaxation dispersion of the δ -positions of V488 at 20° C and 30° C (Figure S4). Whether this is truly reflective of functional dynamics would require a full kinetic analysis of error incorporation in ϕ 12 P2 as demonstrated in 3Dpol together with a complete dynamic analysis of the elongating complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) The structural domains of the RdRps (P2) from cystoviruses $\phi 12$ (left) and $\phi 6$ (right). The domains are as follows – fingers ($\phi 12$: 35-78, 147-303, 358-420; $\phi 6$: 1-30, 104-276, 333-397; red), thumb ($\phi 12$: 82-134, 527-600; $\phi 6$: 37-91, 518-600; blue), palm ($\phi 12$: 304-357, 421-526; $\phi 6$: 277-332, 398-517) and the C-terminal domain (CTD; $\phi 12$: 601-659; $\phi 6$: 601-664; yellow). (B) The conserved sequence motifs A ($\phi 12$: 349-359; $\phi 6$: 324-334; orange-red), B ($\phi 12$: 416-436; $\phi 6$: 394-413; blue), C ($\phi 12$: 461-477, $\phi 6$: 445-461; coral green), D ($\phi 12$: 487-500; $\phi 6$: 475-488; purple), E ($\phi 12$: 505-517; $\phi 6$: 493-505; pink) and F ($\phi 12$: 295-299; $\phi 6$: 268-272; orange). (C) The methyl groups of Ile ($\delta 1$, both $\phi 12$ and $\phi 6$, red), Leu ($\phi 12$ only, coral green), Val ($\phi 12$ only, pink) and Met ($\phi 12$ only, orange) for which resonance assignments are available are shown on the three-dimensional structures of the cystoviral P2 proteins. Unassigned Ile ($\delta 1$ only), Leu, Val and Met methyl groups are shown as grey spheres. The arrows on the top panel depict the entrance to the template entry tunnel. The substrate NTPs enter through a tunnel at the back that is almost orthogonal to the template entry tunnel.

Figure 2.

(A) Close-ups of the “allowed” (left) and “forbidden” spectra for M318 and I168 showing the change in peak intensities with relaxation delay (T). The “forbidden” spectra have been contoured at a 3.8-fold lower level than the “allowed” spectra. The forbidden spectrum for $T = 1 \text{ ms}$ uses double the number of scans as the corresponding allowed spectrum. (B) The experimental intensity ratios corresponding to the spectra shown in (B) (solid circles, M318, black; I168, red) and the fits (solid lines) to Equation 2 are shown on the bottom panel.

Figure 3.

Residues that show statistically significant ΔS^2_{axis} values in the presence of Mg^{2+} (compared to the unliganded state of $\phi 12$ P2; top panel), $\text{Mg}^{2+}/\text{GMPCPP}$ (compared to the Mg^{2+} -loaded state of $\phi 12$ P2; middle panel) and for the *ts*-mutant (the T425I mutant compared to wild-type, unliganded $\phi 12$ P2; bottom panel). ΔS^2_{axis} between states i and j are considered to be statistically significant when $\Delta S^2_{\text{axis}} = |S^2_{\text{axis}}(i) - S^2_{\text{axis}}(j)| > \sigma S^2_{\text{axis}}(i) + \sigma S^2_{\text{axis}}(j) + 0.03$; $\sigma S^2_{\text{axis}}(i)$ is the error in the S^2_{axis} value for the i^{th} state. Remote residues (defined as those that do not have any heavy atoms within 15 Å of any other atom of the binding/mutation site) are labeled. The fonts are colored based on the domain that houses a particular residue – red for fingers, green for palm, blue for thumb and yellow for C-terminal. Residues that comprise the secondary Mg^{2+} -binding site (G348, E503, V507 and D470) are shown and colored green in the top panel. The catalytic residues (D349, D469 and D470) are colored cyan and the two GTP molecules are colored purple and magenta in the middle panel. The site of mutation (T425I) is colored orange (and labeled in black) in the bottom panel.

Figure 4.

(A) Chemical shift differences for methyl positions between wild-type $\phi 12$ P2 and the *ts*-mutant, are shown. Residues that display significant perturbations ($\delta > 0.04$ ppm) are labeled. The magenta font indicates residues that do not have any heavy atoms within 10 Å of any heavy atoms of the mutation site. (B) Plot of the chemical shift changes (δ) against the renormalized closeness scores (Π) calculated using Equation 5 for wild-type P2 (black circles) and the *ts*-mutant (open red circles). Residues with significant δ values (> 0.04 ppm) also have large Π values (> 0.5) as indicated by the shaded region on the top right quadrant bound by $\Pi = 0.5$ and $\delta = 0.04$ ppm (indicated by the dashed lines).

Figure 5.
(A) Distribution of Σ values (calculated using Equation 6) ~ 0.25 in P2. **(B)** Distribution of Σ values ~ 0.75 . **(C)** Distribution of Σ values in the conserved RdRp sequence motifs (A-F) that form the catalytic site. In all cases the P2 proteins from $\phi 12$ and $\phi 6$ cystoviruses are shown in the left and right panels, respectively.

Table 1

S^2_{axis} values for various residue types in $\phi 12$ P2

Domain	Residue	Apo	Mg ²⁺	Mg ²⁺ /GMPCPP	δs -mutant
Fingers	Ile	0.63±0.27 (10)	0.67±0.32 (10)	0.61±0.27 (10)	0.66±0.28 (10)
	Leu	0.69±0.30 (22)	0.74±0.29 (22)	0.69±0.29 (21)	0.73±0.32 (21)
	Val	0.71±0.32 (20)	0.74±0.33 (22)	0.66±0.32 (19)	0.79±0.31 (21)
	Met	0.31±0.18 (6)	0.38±0.23 (7)	0.36±0.23 (7)	0.33±0.17 (6)
	Total	58	61	57	58
Thumb	Ile	0.72±0.20 (7)	0.72±0.18 (7)	0.68±0.20 (6)	0.76±0.21 (7)
	Leu	0.55±0.24 (11)	0.59±0.29 (11)	0.55±0.26 (11)	0.55±0.31 (12)
	Val	0.87±0.12 (9)	0.93±0.16 (11)	0.84±0.13 (9)	0.97±0.22 (9)
	Met	0.48 (1)	0.60 (1)	None	None
	Total	28	30	26	28
Palm	Ile	0.87±0.12 (6)	0.93±0.08 (6)	0.82±0.07 (6)	0.84±0.27 (7)
	Leu	0.55±0.29 (15)	0.63±0.26 (14)	0.57±0.27 (15)	0.59±0.28 (14)
	Val	0.75±0.29 (11)	0.82±0.31 (14)	0.75±0.28 (15)	0.77±0.32 (12)
	Met	0.43±0.18 (4)	0.44±0.18 (5)	0.45±0.17 (5)	0.45±0.18 (5)
	Total	36	39	41	38
CTD	Ile	0.57±0.43 (2)	0.70±0.57 (2)	0.54±0.39 (2)	0.71±0.59 (2)
	Leu	None	0.78±0.09 (2)	0.74±0.03 (2)	None
	Val	0.50±0.38 (3)	0.61±0.36 (4)	0.50±0.36 (3)	0.51±0.36 (4)
	Met	0.43 (1)	0.58±0.12 (2)	0.54±0.12 (2)	0.65±0.19 (2)
	Total	6	10	9	8
Overall ^a	Ile	0.71±0.24 (26)	0.75±0.26 (26)	0.68±0.22 (25)	0.74±0.27 (27)
	Leu	0.60±0.29 (51)	0.67±0.28 (51)	0.61±0.27 (52)	0.63±0.31 (50)
	Val	0.72±0.30 (45)	0.78±0.31 (53)	0.70±0.29 (48)	0.78±0.32 (47)
	Met	0.37±0.17 (12)	0.44±0.20 (15)	0.42±0.19 (14)	0.42±0.20 (13)
	Total	134	145	139	137

^aTotal for all Ile, Leu, Val and Met residues including those that do not belong to the canonical domains. Average values and the corresponding standard deviations are calculated over all residues of a particular type in a given structural domain.

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Table 2

Σ values for the viral RdRp sequence motifs

Motif	ϕ12	Apo	Mg ²⁺	Mg ²⁺ /GMPCPP	ts-mutant	ϕ6	Apo	Mg ²⁺
F	V299, γ1/2	0.20	0.27	0.22	0.28			
F	V299, γ2/1	0.24	0.29	0.26				
A	V350, γ1/2	0.77	0.60		0.62			
A	V350, γ2/1			0.74				
B	<i>I425, δ1</i>				<i>0.00</i>			
B	L427, δ1/2	0.67	0.54	0.56	0.55			
B	L427, δ2/1	0.85	0.76	0.68	0.72			
B	V431, γ1/2	0.60	0.54	0.62	0.67	I408, δ1	0.72	0.75
B	V431, γ2/1		0.62	0.68				
B	V434, γ1/2		0.65	0.70	0.56			
B	V434, γ2/1	0.62	0.81	0.75	0.75			
B	I435, δ1	0.80	0.74	0.77				
C						I446, δ1	0.92	0.88
C	I465, δ1	0.48	0.64	0.65	0.68	I449, δ1	0.80	0.80
C	I472, δ1	0.64	0.71	0.62	0.78			
C	V473, γ1/2	0.14	0.09	0.06	0.17			
D	L487, δ1/2	0.13		0.17	0.24			
D	L488, δ1/2	0.16						
D	L488, δ2/1	0.10	0.12	0.07	0.22			
D	M497, ε1	0.65	0.36	0.36	0.46			
D	V500, γ1/2	0.55	0.48	0.43	0.46	I488, δ1	0.04	0.08
E						I500, δ1	0.03	0.07
E	L515, δ1/2	0.34	0.30	0.25	0.39			

The corresponding residues for ϕ6 and ϕ12 P2 in the table have been aligned based on their sequential position on their respective motifs (below; probe methyls have been indicated by the green font. The conserved residues are shown in bold font. I425 (italicized) that exists only for the ts-mutant (I425 in wild-type) is the most dynamic of all Ile residues in the mutant.

Motif A

ϕ12: 349**D** **V**SSYDHSE³⁵⁹

ϕ6: 324**D**VSD**H**D-TFWPG³³⁴

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Motif B
φ12: 416AGNRSGHAFTS **L**FAK **V**WK **V**LD⁴³⁶
φ6: 393SGQGATDLMGTLMS **I**TYLV⁴¹³
Motif C
φ12: 461PFGC **I**NNGDDE **I**VWFKS⁴⁷⁷
φ6: 445E **I**RQ **I**SKSDDAMLGWTK⁴⁶¹
Motif D
φ12: 487 **L**L₁ETQPQEQR **M**FK **V**₅₀₀
φ6: 475 **L**KEGKVNFPYMK **I**₄₈₈
Motif E
φ12: 505GAVFSGSVYQ **L**IG⁵¹⁷
φ6: 493GGAFLGD **I**LLYDS⁵⁰⁵
Motif F
φ12: 295 **R**TR **L**₂₉₉
φ6: 268 **R**RTA²⁷²