Published in final edited form as:

DNA Repair (Amst). 2015 May ; 29: 65–73. doi:10.1016/j.dnarep.2015.01.012.

Steric gate residues of Y-family DNA polymerases DinB and pol kappa are crucial for dNTP-induced conformational change

Philip Nevin, John R. Engen, and Penny J. Beuning*

Department of Chemistry and Chemical Biology, 360 Huntington Avenue, Northeastern University, Boston, MA 02115, USA

Abstract

Discrimination against ribonucleotides by DNA polymerases is critical to preserve DNA integrity. For many DNA polymerases, including those of the Y family, rNTP discrimination has been attributed to steric clashes between a residue near the active site, the steric gate, and the 2'-hydroxyl of the incoming rNTP. Here we used hydrogen/deuterium exchange (HDX) mass spectrometry (MS) to probe the effects of the steric gate in the Y-family DNA polymerases *Escherichia coli* DinB and human DNA pol κ . Formation of a ternary complex with a G:dCTP base pair in the active site resulted in slower hydrogen exchange relative to a ternary complex with G:rCTP in the active site. The protection from exchange was localized to regions both distal and proximal to the active site, suggesting that DinB and DNA pol κ adopt different conformations depending on the sugar of the incoming nucleotide. In contrast, when the respective steric gate residues were mutated to alanine, the differences in HDX between the dNTP- and rNTP-bound ternary complexes were attenuated such that for DinB(F13A) and pol κ (Y112A), ternary complexes with either G:dCTP or G:rCTP base pairs had similar HDX profiles. Furthermore, the HDX in these ternary complexes resembled that of the rCTP-bound state rather than the dCTP-bound state of the wild-type enzymes. Primer extension assays confirmed that DinB(F13A) and pol κ (Y112A) do not discriminate against rNTPs to the same extent as the wild-type enzymes. Our observations indicate that the steric gate is crucial for rNTP discrimination because of its role in specifically promoting a dNTP-induced conformational change and that rNTP discrimination occurs in a relatively closed state of the polymerases.

Keywords

DNA replication; nucleotide selection; enzyme specificity; ribonucleotide; hydrogen exchange mass spectrometry

© 2015 Published by Elsevier B.V.

*Corresponding author: Tel.: +1 617 373 2865, beuning@neu.edu.

Conflict of interest statement

JRE is a paid consultant of the Waters Corporation.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

DNA polymerases (pols) are able to select the correct substrate from a pool of nucleotides with similar chemical properties. The ability of DNA pols to discriminate against ribonucleoside triphosphates (rNTPs) is essential since the cellular rNTP concentration is much higher than the dNTP concentration and ribonucleotide incorporation into DNA leads to replication stress and genomic instability [1]. While DNA pols from different families differ widely in their dNTP specificities and misincorporation rates [2], both high- and low-fidelity DNA pols efficiently discriminate against rNTPs [3]. Exceptions include the X-family DNA pols terminal deoxynucleotidyl transferase [4, 5], pol μ [5], and yeast pol IV [6], which have been shown to discriminate against rNTPs by only ~10-fold or less. Many DNA pols form correct Watson-Crick base pairs using conformational changes prior to the chemistry step [7–9] whereas sugar discrimination has been attributed to a simple steric clash between an active site residue, the steric gate, and the 2'-OH group on the ribose of an incoming rNTP [3] (Figure 1a).

The steric gate model is supported by mutagenesis studies of DNA pols from both low- and high-fidelity families [10–24], which have shown that mutation of the steric gate, which is typically a bulky active site residue, to a smaller residue significantly decreases discrimination against ribonucleotides. Moreover, introduction of a bulky residue (G433Y) in the active site of pol μ , which only weakly discriminates against rNTPs, resulted in increased sugar discrimination [25]. Structural studies have also shown that mutation of the steric gate residue results in accommodation of rNTPs in the active site of high- [18] and low-fidelity [26] DNA pols. However, the steric gate variants still prefer dNTPs over rNTPs and altered selectivity is generally accompanied by a decrease in primer extension activity, indicating that sugar selectivity and dNTP insertion mechanism are not independent. Several structural and kinetic studies indicate a more nuanced model in which conformational changes are involved in the sugar specificity of DNA pols [27, 28]. The steric gate residues have also been demonstrated to be important for lesion bypass in some Y family DNA polymerases [20, 22, 29, 30].

In contrast to high-fidelity DNA pols, distinct conformations of low-fidelity DNA pols upon binding correct incoming nucleotides have been difficult to identify. For example, crystal structures of the ternary complex (pol-DNA-nucleotide) of *E. coli* Y-family DNA pol DinB with mismatched or complementary base pairs in the active site are highly similar [31]. The similarities of crystal structures of Y-family pols in various states in the catalytic cycle have led to the hypothesis that these enzymes do not undergo nucleotide-induced conformational changes and exist in a closed conformation prior to the chemistry step [32, 33]. This is in contrast to many high-fidelity pols, which undergo a nucleotide-induced conformational change prior to the chemistry step, that in some cases is rate-limiting [7].

The steric gate of DinB has been identified as phenylalanine 13 (F13) and when mutated to a smaller residue results in an enzyme with reduced rNTP discrimination but also reduced primer extension activity [20]. Similar observations have been made for the human DinB ortholog DNA polymerase κ (pol κ) when the steric gate tyrosine 112 (Y112) was mutated to a smaller residue [24]. To gain further insight into the role of conformational dynamics in

the mechanism of rNTP discrimination by DinB and pol κ , we have probed substrate-dependent changes in the rate of hydrogen exchange in DinB and pol κ using hydrogen/deuterium exchange (HDX) coupled with mass spectrometry (MS). HDX MS probes the hydrogen bonding and solvent accessibility of the protein backbone amide hydrogens, and is well suited for characterizing structural changes of proteins in solution [34–36]. Disruption of H-bonds, e.g. due to backbone fluctuations, structural rearrangements, or ligand binding, increases the probability of exchange for the hydrogen involved in those bonds, resulting in different HDX patterns for different protein conformations or states [37, 38].

We find here that the extent of deuterium incorporation into DinB and pol κ was lower in ternary complexes with the complementary dNTP than in ternary complexes with the complementary rNTP, indicating that only a nucleotide with deoxyribose gives the maximal protection from exchange that we observed. For steric gate variants, the HDX was similar in the presence of both dNTPs and rNTPs. Moreover, most of the backbone of the dNTP- or rNTP-bound ternary complex of the steric gate variants had HDX similar to that of the rNTP-bound ternary complex of the respective WT pols. Our observations suggest that the steric gate prevents rNTP incorporation and is crucial for the promotion of a dNTP-induced closed conformation of the DinB and pol κ .

2. Materials and Methods

2.1 Materials

E. coli DinB and human pol κ (residues 19–526 preceded by GPGS) variants were expressed and purified as described [39, 40]. The DinB(F13A) and pol κ (Y112A) variants were created by site-directed mutagenesis procedures using primers with the following sequences 5'-GAT ATG GAC TGC TTT GCC GCC GCA GTG-3' and 5'-GAC ATG GAT GCT TTC GCT GCA GCT GTA G -3', respectively, and a QuikChange kit (Agilent). DNA was from Eurofins Operon. Template DNA used in primer extension assays, HDX experiments, and thermal shift assays had the sequence 5'-CCT AGG CGT CCG GCA AGC-3' and the primer sequence was 5'-GCT TGC CGG ACG C-3'. For HDX and thermal shift experiments that included nucleotides, a 2',3'-dideoxy-terminated primer was used to avoid nucleotide incorporation. Oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis and the crush and soak method [41].

2.2 HDX MS

Proteins (25 μ M) were allowed to equilibrate in the presence or absence of primer/template DNA (46 μ M) and dCTP or rCTP (5 mM) in 20–40 μ L binding buffer (25 mM Hepes, pH 7.5, 20 mM NaCl, 5 mM MgSO₄, and 5 mM dithiothreitol) for at least 20 min at 22 °C. HDX reactions were initiated by diluting 3 μ L of the mixture 17-fold with D₂O buffer (25 mM Hepes, pH 7.5, 20 mM NaCl, 5 mM MgSO₄) at 22 °C and quenched after defined periods of time (10 s, 1 min, 10 min, 1 h, and 4 h) by addition of an equal volume of ice-cold quench buffer (0.1 M sodium phosphate, 4 M guanidinium HCl, 0.05 M TCEP, pH 2.0). The final pH of the quenched reactions was 2.7.

Following quenching, the HDX reactions were immediately injected into a nanoACQUITY system with HDX technology for UPLC separation (Waters) [42] and passed through a 2.1

mm × 50 mm stainless-steel column packed with immobilized pepsin [43] with a flow rate of 100 µL/min in 0.1% formic acid at 15 °C. Peptides were trapped and desalted on an ACQUITY UPLC BEH C18 1.7 µm 2.1 mm × 5 mm VanGuard Pre-column (Waters) kept at 0 °C. After 4 min, the trap was placed in line with an ACQUITY UPLC BEH C18 1.7-µm 1.0 mm × 100 mm column (Waters) and a 7 min linear gradient (8 – 40% acetonitrile, 0.1% formic acid, pH 2.5) at a flow rate of 40 µL/min was used to separate the peptides at 0 °C and direct them into a Synapt G1 mass spectrometer with an electrospray ionization source (Waters). Mass spectra were acquired in positive ion mode over an m/z range of 50 – 1,900 under optimized conditions. Continuous lock-mass correction was performed using Glu-fibrinogen B peptide standard (Sigma).

2.3 Peptide identification and data analysis

Peptides in triplicate undeuterated control samples were identified by a combination of accurate mass and collision-induced dissociation in data-independent acquisition mode (MS^E) [44] aided by PLGS software (Waters). Only peptides identified in at least two replicates were included in the analysis. Mass spectra of undeuterated and deuterated peptides at different time points were extracted and analyzed using DynamX 2.0 (Waters). Relative deuterium levels were calculated by subtracting the centroid of the isotope distribution of the undeuterated peptide from that of deuterated peptides. The deuterium levels were not corrected for back-exchange and are therefore reported as relative [38]. The fractional relative deuterium uptake was calculated by dividing the relative deuterium uptake of each peptide by its number of backbone amides after subtracting one for the N-terminus and one for each proline residue [35]. All HDX-MS experiments presented here represent at least two replicates. A 98% confidence interval for the mean relative deuterium uptake of ±0.6 Da was calculated as described [45] and used as a threshold for significance, i.e. differences larger than 0.6 Da were considered significant at 98% confidence.

2.4 Primer extension assays

Primer extension assays were carried out as described previously [46] using ³²P-labeled 13-mer primer annealed to 18-mer template. Reactions contained a final concentration of 10 nM DNA polymerase, 100 nM primer/template DNA, 1 mM dNTPs or rNTPs, 7.5 mM MgSO₄, 30 mM Hepes (pH 7.5), 20 mM NaCl, 2 mM β-mercaptoethanol, 1% (w/v) bovine serum albumin, and 4% glycerol. Reaction products were separated by denaturing 16% polyacrylamide gel electrophoresis and analyzed by phosphorimaging.

2.5 Thermal shift assays

Samples containing 3 µM protein in the presence or absence of primer/template DNA (2-fold molar excess) and dNTP (1 mM) in 15 µL of assay buffer (25 mM Hepes, pH 7.5, 20 mM NaCl, 5 mM MgSO₄, and 3 mM dithiothreitol) were incubated for 20 min at room temperature before addition of 0.5 µL of Sypro Orange (Invitrogen) to a final concentration of 10X. Samples were analyzed using a CFX 96 Real-Time system (Bio-Rad) as described [47].

3. Results

3.1 Mutation of the steric gate residue modestly affects hydrogen exchange of DinB and pol κ in the absence of substrates

To determine the conformational dynamics of DinB and pol κ and their respective steric gate variants, DinB(F13A) and pol κ (Y112A), we measured the amount of deuterium incorporation into the protein backbone over time using mass spectrometry. The sequence coverage of DinB and pol κ was 86% and 82%, respectively, after analyzing all the HDX data (Figure S1). We mapped the peptide-level HDX data onto the crystal structure of DinB [31] and pol κ [48] to visualize the local exposure to deuterium exchange of the free enzymes (Figure 1b). We found that the most stable regions of DinB and pol κ were the palm and LF domains, which remained relatively protected from HDX over several hours of exchange. In contrast, the fingers domains in DinB and pol κ were rapidly deuterated. The pol κ N-clasp was unprotected from HDX and was almost fully deuterated after 10 s of HDX, indicating that this region is unstructured in the absence of DNA, as has been suggested [48]. We measured the difference in deuterium uptake between the WT and the steric gate variants and displayed the difference according to a color gradient for each peptide and time point as shown in Figure 1c. The complete HDX data sets for all DinB and pol κ peptides are provided in Table S1 and Table S2, respectively. Mutating the DinB steric gate residue F13 to alanine did not alter the HDX rates for the majority of residues of DinB (Figure 1c, left) or change the thermodynamic stability (Figure 2, top). For pol κ , a number of regions in proximity of the 112 position were more exposed in Y112A than in the WT, while other regions were more protected (Figure 1c, right). The melting temperature of pol κ (Y112A) (41.1 °C) was 2.5 °C lower than that of WT pol κ (43.6 °C; Figure 2, bottom). While most regions of DinB and pol κ did not exhibit altered HDX behavior due to the steric gate mutation, DinB residues 77–88 and the corresponding region of pol κ , residues 165–180 and adjacent residues 117–135, showed increased HDX due to mutation of the steric gate to a smaller residue (Figure 1d). This region (DinB 77–88; pol κ 165–180) is part of an α -helix located on the outer edge of the palm domain (Figure 1e) and contains residue Y79 (pol κ Y174), which appears to stack with F13 (pol κ Y112) in crystal structures [31, 48]. The increase in HDX in this region due to the F13A mutation in DinB and Y112A mutation in pol κ is consistent with the loss of this stacking interaction. Pol κ residues 222–243 (palm domain loop insertion), 345–364 (thumb), and 442–457 (LF) exhibited significantly less deuterium uptake in the Y112A variant than in WT pol κ (Figure 1c and 1f).

3.2 DinB and pol κ are protected from hydrogen exchange by G:dCTP but not G:rCTP

To probe the ability of DinB and pol κ to discriminate against rNTP incorporation into DNA, we first performed primer extension assays with either only dNTPs or only rNTPs. DinB efficiently extended primers using dNTPs but was only very weakly able to extend primers in the presence of only rNTPs (Figure 3a), confirming that DinB efficiently discriminates against rNTP incorporation. We made similar observations for pol κ although pol κ extended primers using only rNTPs more efficiently than DinB, weakly inserting one nucleotide (Figure 3b). While DinB extended primers to the end of the template, we did not detect full-length products for pol κ under our reaction conditions. For both DinB and pol κ , the steric gate variant extended primers using dNTPs with similar efficiency as when using

rNTPs (Figure 3a and b). However, unlike the WT pols, which produced mainly 16- or 17-mer products with dNTPs, the steric gate variants were less efficient and inserted only a few nucleotides to produce mainly 14- or 15-mer products under the same conditions. This indicates that while the DinB(F13A) and pol κ (Y112A) mutations result in pol variants with diminished sugar discrimination, these variants are also less efficient at primer extension using dNTPs in relation to the respective WT proteins. These observations are consistent with previously reported observations for DinB(F13V) [20] and pol κ (Y112A) [24].

We performed a thermal shift assay to determine substrate-dependent changes in the thermodynamic stability of DinB and pol κ . The WT DinB ternary complex with a G:dCTP base pair had a melting temperature (51.6 °C) that was 4.0 °C higher than that of a ternary complex with a G:rCTP base pair (47.6 °C; Figure 2, top). In contrast, DinB(F13A) ternary complexes had the same melting temperature (46.8 °C) with either dCTP or rCTP as incoming nucleotide. Furthermore, the melting temperature of the DinB(F13A) complexes was similar to that of the WT DinB complex with the G:rCTP base pair (47.6 °C). For WT pol κ , the ternary complex with G:dCTP had a melting temperature (55.3 °C) that was 6.3 °C higher than that of the ternary complex with a G:rCTP base pair (49.0 °C; Figure 2, bottom). For pol κ (Y112A), both the dCTP- and rCTP-bound complexes had melting temperatures (50.2 °C and 47.6 °C) similar to that of the rCTP-bound WT ternary complex (49.0 °C).

To gain further insights into the difference in substrate specificity between the WT pols and their steric gate variants and to determine how the conformational dynamics of the pols depend on the identity of the sugar of the incoming nucleotide, we compared the HDX for pol-DNA-dCTP and pol-DNA-rCTP using both wild-type DinB and the F13A variant. These experiments were performed with a DNA template that had G as the template base and a dideoxy-terminated primer to avoid nucleotide incorporation. For each comparison, the deuterium levels of each peptide and time point for one state were subtracted from the second state. Figure 4a shows the difference in deuterium uptake between the dCTP- and rCTP-bound ternary complexes of DinB (left) and DinB(F13A) (right). Several regions of the dCTP-bound state of DinB were protected from exchange in relation to the rCTP-bound ternary complex, indicating structural differences between the two different ternary complexes. Prominent protection was seen in the fingers domain (residues 16–61; Figure 4), where several backbone amides (differences of >3 Da in some peptides) in the active site loops were protected. While the HDX for most of the palm domain was similar in the dCTP- and rCTP-bound ternary DinB complexes, palm residues 148–162 showed significant protection from HDX in the dCTP-bound complex (Figure 4).

In addition to regions surrounding the active site, several regions distal to the nucleotide binding site exhibited protection from HDX in the dCTP-bound state in relation to the rCTP-bound state. These regions include parts of the thumb domain (residues 173–192), which make contact with the DNA backbone in the minor groove, parts of the LF domain (residues 240–251 and 283–307), which make contact in the major groove, and the linker between the thumb and LF domains (Figure 4c). Because these regions do not make contacts with the incoming nucleotide, the HDX differences in these distal regions indicate that DinB adopts different global conformations when bound to dNTP than when bound to rNTP.

Specifically, the linker connecting the thumb and LF domains, as well as the two outer β strands in the LF domain β sheet were more protected upon dCTP binding than rCTP binding. For nearly all peptides exhibiting differences, there was less HDX in the dCTP-bound ternary complex of DinB than in the corresponding rCTP complex (Figure 4a, left). Specifically, the fingers, thumb, and LF are strongly protected from exchange in the presence of G:dCTP relative to G:rCTP.

Similarly to DinB, several regions of pol κ both proximal and distal to the incoming nucleotide were protected in the dCTP-bound ternary complex relative to the rCTP-bound complex (Figure 5a–c), indicative of structural differences between the dNTP- and rNTP-bound ternary complexes. Modest protection was observed in the fingers domain (residues 115–180) while stronger protection was observed in parts of the palm (residues 320–332), thumb (residues 345–364), LF (residues 442–477), and N-clasp (residues 54–67). It is striking that the deoxyribose-dependent protection in the palm, thumb, and LF virtually mirrors the protection pattern observed for the corresponding regions of DinB (compare Figure 4b and 5b). The HDX differences in DinB and pol κ due to the identity of the sugar of the incoming nucleotide correlate with the differences in the ability of DinB and pol κ to extend primers using either dNTPs or rNTPs (Figure 3), suggesting that the strong protection from exchange observed is related to sugar discrimination.

We hypothesized that mutation of the steric gate residue to alanine would result in dCTP- and rCTP-bound ternary complexes with similar HDX profiles. To test this, we repeated the HDX-MS experiments with DinB(F13A) and pol κ (Y112A) ternary complexes. In contrast to wild-type DinB, HDX of DinB(F13A)-DNA-dCTP was highly similar to that of DinB(F13A)-DNA-rCTP (Figure 4a, right), consistent with the similar thermodynamic stability of the two complexes (Figure 2), and the lack of sugar discrimination during primer extension (Figure 3a). Similar results were obtained for pol κ (Y112A) (Figure 2, 3b, and 5a), although certain regions of pol κ (Y112A) exhibited protection from HDX in the dCTP-bound complex in relation to the rCTP-bound complex, mainly in the N-clasp.

3.3 Hydrogen exchange of the ternary DinB(F13A) and pol κ (Y112A) complexes resembles the rCTP-bound ternary complexes

Since the substrate-dependent protection from HDX in DinB and pol κ correlated with primer extension activity, and because mutation of the steric gate modestly reduced the efficiency of DNA synthesis by DinB and pol κ but did not abolish it (Figure 3), HDX of the dCTP- and the rCTP-bound ternary complexes of DinB(F13A) and pol κ (Y112A) might be expected to resemble that of the respective dCTP-bound ternary WT complexes. However, the HDX of the two ternary DinB(F13A) complexes resembled that of the ternary complex of WT DinB bound to rCTP rather than that of the dCTP-bound complex (Figure 6a). Most peptides of DinB-DNA-rCTP exhibited HDX highly similar to DinB(F13A)-DNA-rCTP as well as to DinB(F13A)-DNA-dCTP; however, a few differences were observed. Peptides in the fingers (residues 44–61), thumb (residues 174–192), and LF (residues 240–251), showed slight exposure in the rCTP-bound ternary complex of WT DinB in relation to the two ternary complexes of DinB(F13A) (Figure 6b). For pol κ , the comparison between the ternary Y112A complexes and the WT rCTP complex is not straightforward due to

differences in the intrinsic HDX rates of pol κ (Y112A) and WT pol κ (Figure 1c, right). However, in general the HDX-MS indicates that the dCTP- and rCTP-bound ternary complexes of pol κ (Y112A) are structurally similar to the rCTP-bound WT complex (Figure 6c and 6d).

We identified one region of DinB and pol κ , represented by DinB residues 77–88 and the corresponding pol κ residues 165–180, that exhibited protection from HDX in the rCTP-bound ternary complex of the WT enzymes relative to the corresponding rCTP-bound steric gate variant complexes (Figure 6b and 6d). In addition, only this region exhibited significant protection from HDX in both the dCTP- and rCTP-bound ternary complexes of WT DinB relative to the other complexes (Figure 6b and 6d). As mentioned in section 3.1, this region contains DinB residue Y79 (pol κ Y174) that stacks with F13 (pol κ Y112), which in turn stacks with the incoming nucleotide. Thus, the protection in this region in both the dCTP- and rCTP-bound ternary WT complexes indicate a stabilization of residues 77–88 due to formation of a ternary complex. When the steric gate residue was mutated to alanine, protection was still observed in this region due to formation of the ternary complex, but to a lesser extent than with WT DinB or pol κ . Thus, this region is stabilized by nucleotide binding but maximum protection only occurs when the steric gate is present.

4. Discussion

The steric gate model for rNTP discrimination by DNA polymerases is an example of how a single side chain in an enzyme can govern substrate specificity. Most steric gate variants are reported to have diminished ability to extend primers using dNTPs, suggesting that the steric gate is also important for dNTP incorporation [10–24]. A crystallographic analysis of archaeal DinB ortholog Dpo4 showed that mutation of the steric gate Y12 to alanine allows accommodation of the 2'-OH of rNTPs in the active site [26]. The reduced efficiency in nucleotide incorporation of the Y12A variant was explained by the loss of stacking interactions between Y12 and the sugar ring on the incoming nucleotide. The crystal structures of DinB and pol κ also indicate a stacking network of Y79 (pol κ Y174), F13 (pol κ Y112), and the sugar of the incoming nucleotide; DinB(F13A) and pol κ (Y112A) show both reduced discrimination against rNTPs and reduced primer extension (Figure 3) [20, 31, 48]. Our observation that pol variants with mutated steric gates and therefore diminished sugar discrimination (Figure 3) are protected from hydrogen exchange independent of the sugar of the incoming nucleotide (Figure 4 and 5), is consistent with accommodation of both rNTPs and dNTPs in the active site due to the void resulting from removal of the aromatic side chain of the steric gate residue. In addition, our observation that DinB (F13A) and pol κ (Y112A) ternary complexes with either dNTPs or rNTPs did not exhibit protection from hydrogen exchange to the same extent as the respective dNTP-bound WT ternary complex (Figure 4, 5 and 6) is consistent with the loss of the stacking interaction between the steric gate and the sugar on the incoming nucleotide, by analogy to Dpo4. Thus, the reduced primer extension efficiency is explained by the loss of the steric gate-nucleotide stacking interaction.

As shown in section 3.2, dNTP-bound ternary complexes of DinB and pol κ exhibited protection from HDX relative to the rNTP-bound complexes. The fact that strong protection

occurred in regions distal to the nucleotide binding site (Figure 4 and 5) suggests that the reduction in HDX was caused by a change in conformation rather than a direct ligand interaction. The strong protection in the dNTP-bound ternary complex is consistent with a state in which the fingers, thumb, and LF domains are in a relatively “closed” conformation. For pol κ , our observations additionally indicate that the N-clasp exhibits structural dynamics that depend on the sugar of the incoming nucleotide. The correlation between the sugar-dependent HDX differences for ternary complexes with non-extendable primers and the sugar discrimination during primer extension assays suggests that conformational changes in DinB and pol κ prior to the chemistry step are important for dNTP incorporation and sugar discrimination. Since the HDX data for DinB(F13A) and pol κ (Y112A) ternary complexes suggest that the steric gate variants do not adopt the “closed” conformation of wild-type pols, and DinB(F13A) as well as pol κ (Y112A) do not discriminate against rNTPs during primer extension, sugar discrimination likely takes place in the “closed” conformation prior to the chemistry step. Alternatively, the proposed conformational change itself could act as a sugar-discrimination checkpoint prior to the chemistry step. For DNA pol I (Klenow fragment; KF), stopped-flow and single-molecule studies have indicated that the rate of fingers-closing is reduced by complementary rNTPs in relation to correct dNTPs [27] and that ternary complexes with rNTPs favor an intermediate state (between the open and closed states) [49]. In addition, unlike WT KF, ternary complexes of KF steric gate variant E710A with rNTPs or dNTPs have been shown to favor the intermediate state rather than the closed state [50]. These findings are consistent with our observation that DinB(F13A) and pol κ (Y112A) ternary complexes appear to disfavor the “closed” state that occurs with the WT enzymes and the correct incoming dNTP.

How does dNTP binding in the active site cause reduced HDX in the thumb and LF domains of DinB and pol κ ? It has been demonstrated that mutation of Y79 to a smaller residue reduces the efficiency with which DinB fully extends primers mainly due to increased stalling three bases downstream of the first nucleotide incorporated [51]. Because Y79 is located near the active site, behind F13 with respect to the incoming nucleotide (Figure 1e), and does not make contact with DNA near the +3 position, it was hypothesized that there is communication between the +3 position of the DNA and the active site. The dNTP-induced allosteric changes in HDX that we observed also suggest that there is a signal within the enzyme that depends on the identity of the sugar on the nucleotide in the active site. It is likely that the stacking interactions between the dNTP, F13, and Y79 in DinB (Y112 and Y174 in pol κ), which may not occur with rNTPs due to the steric clash between DinB F13 (pol κ Y112) and the 2'-hydroxyl on rNTP, propagate a signal through the palm domain in the form of a structural rearrangement. A number of interactions connect the LF and the thumb, which both exhibited allosteric nucleotide-dependent HDX changes, to the palm domain and the +3 position of the DNA. First, DinB thumb residues 173–192 (pol κ 345–364), which exhibit allosteric HDX changes, make contact with the primer strand at the +3 position and interact with palm residues 148–162 (pol κ 320–332), which appear to make contact with the incoming nucleotide (Figures 4c, 5c). This network of interactions could communicate a signal in the form of changes in structural dynamics between the thumb and the active site. Second, a linker (DinB residues 228–241; pol κ residues 401–414) near the +3 position in the major groove connects the thumb and LF domains. This linker in DinB as

well as the LF in both DinB and pol κ showed allosteric HDX differences between the dNTP- and rNTP-bound complexes. A signal could be propagated either between the thumb and LF via the linker or through DNA between the active site and the LF. Our observations suggest that DinB and pol κ undergo a dNTP-induced and steric gate-dependent structural change important for substrate specificity and catalysis; indeed, when the steric gate residue was mutated to alanine, the HDX indicated that DinB and pol κ did not undergo the proposed closing transition (Figure 4 and 5). Thus alterations to the aforementioned network of interactions may alter the activity of DinB or pol κ .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We give special thanks to Prof. Fred Guengerich (Vanderbilt University) for providing the pol κ expression plasmid. This work was supported by generous financial support from the NSF (CAREER Award, MCB-0845033 to PJB), the NIH (R01-GM101135 to JRE), a research collaboration with the Waters Corporation, the American Cancer Society (Research Scholar Grant RSG-12-161-01-DMC to PJB), Research Corporation for Science Advancement (Cottrell Scholar Award to PJB), and the NU Office of the Provost.

Abbreviations

dNTP	deoxyribonucleoside triphosphate
rNTP	ribonucleoside triphosphate
pol	polymerase
HDX	hydrogen/deuterium exchange
MS	mass spectrometry
LF	little finger
WT	wild-type

References

1. Nick McElhinny SA, Kumar D, Clark AB, Watt DL, Watts BE, Lundstrom EB, Johansson E, Chabes A, Kunkel TA. Genome instability due to ribonucleotide incorporation into DNA. *Nat Chem Biol.* 2010; 6:774–781. [PubMed: 20729855]
2. McCulloch SD, Kunkel TA. The fidelity of DNA synthesis by eukaryotic replicative and translesion synthesis polymerases. *Cell Res.* 2008; 18:148–161. [PubMed: 18166979]
3. Brown JA, Suo Z. Unlocking the sugar “steric gate” of DNA polymerases. *Biochemistry.* 2011; 50:1135–1142. [PubMed: 21226515]
4. Boule JB, Rougeon F, Papanicolaou C. Terminal deoxynucleotidyl transferase indiscriminately incorporates ribonucleotides and deoxyribonucleotides. *J Biol Chem.* 2001; 276:31388–31393. [PubMed: 11406636]
5. Nick McElhinny SA, Ramsden DA. Polymerase mu is a DNA-directed DNA/RNA polymerase. *Mol Cell Biol.* 2003; 23:2309–2315. [PubMed: 12640116]
6. Bebenek K, Garcia-Diaz M, Patishall SR, Kunkel TA. Biochemical properties of *Saccharomyces cerevisiae* DNA polymerase IV. *J Biol Chem.* 2005; 280:20051–20058. [PubMed: 15778218]

7. Joyce CM, Benkovic SJ. DNA polymerase fidelity: kinetics, structure, and checkpoints. *Biochemistry*. 2004; 43:14317–14324. [PubMed: 15533035]
8. Berdis AJ. Mechanisms of DNA polymerases. *Chem Rev*. 2009; 109:2862–2879. [PubMed: 19489544]
9. Federley RG, Romano LJ. DNA polymerase: structural homology, conformational dynamics, and the effects of carcinogenic DNA adducts. *J Nucleic Acids*. 2010
10. Astatke M, Ng K, Grindley ND, Joyce CM. A single side chain prevents *Escherichia coli* DNA polymerase I (Klenow fragment) from incorporating ribonucleotides. *Proc Natl Acad Sci U S A*. 1998; 95:3402–3407. [PubMed: 9520378]
11. Bonnin A, Lazaro JM, Blanco L, Salas M. A single tyrosine prevents insertion of ribonucleotides in the eukaryotic-type phi29 DNA polymerase. *J Mol Biol*. 1999; 290:241–251. [PubMed: 10388570]
12. Gao G, Orlova M, Georgiadis MM, Hendrickson WA, Goff SP. Conferring RNA polymerase activity to a DNA polymerase: a single residue in reverse transcriptase controls substrate selection. *Proc Natl Acad Sci U S A*. 1997; 94:407–411. [PubMed: 9012795]
13. Cases-Gonzalez CE, Gutierrez-Rivas M, Menendez-Arias L. Coupling ribose selection to fidelity of DNA synthesis. The role of Tyr-115 of human immunodeficiency virus type 1 reverse transcriptase. *J Biol Chem*. 2000; 275:19759–19767. [PubMed: 10748215]
14. Patel PH, Loeb LA. Multiple amino acid substitutions allow DNA polymerases to synthesize RNA. *J Biol Chem*. 2000; 275:40266–40272. [PubMed: 11005812]
15. Yang G, Franklin M, Li J, Lin TC, Konigsberg W. A conserved Tyr residue is required for sugar selectivity in a Pol alpha DNA polymerase. *Biochemistry*. 2002; 41:10256–10261. [PubMed: 12162740]
16. Brown JA, Fiala KA, Fowler JD, Sherrer SM, Newmister SA, Duym WW, Suo Z. A novel mechanism of sugar selection utilized by a human X-family DNA polymerase. *J Mol Biol*. 2010; 395:282–290. [PubMed: 19900463]
17. Kasiviswanathan R, Copeland WC. Ribonucleotide discrimination and reverse transcription by the human mitochondrial DNA polymerase. *J Biol Chem*. 2011; 286:31490–31500. [PubMed: 21778232]
18. Cavanaugh NA, Beard WA, Batra VK, Perera L, Pedersen LG, Wilson SH. Molecular insights into DNA polymerase deterrents for ribonucleotide insertion. *J Biol Chem*. 2011; 286:31650–31660. [PubMed: 21733843]
19. DeLucia AM, Grindley ND, Joyce CM. An error-prone family Y DNA polymerase (DinB homolog from *Sulfolobus solfataricus*) uses a 'steric gate' residue for discrimination against ribonucleotides. *Nucleic Acids Res*. 2003; 31:4129–4137. [PubMed: 12853630]
20. Jarosz DF, Godoy VG, DeLaney JC, Essigmann JM, Walker GC. A single amino acid governs enhanced activity of DinB DNA polymerases on damaged templates. *Nature*. 2006; 439:225–228. [PubMed: 16407906]
21. Sherrer SM, Beyer DC, Xia CX, Fowler JD, Suo Z. Kinetic basis of sugar selection by a Y-family DNA polymerase from *Sulfolobus solfataricus* P2. *Biochemistry*. 2010; 49:10179–10186. [PubMed: 20973506]
22. Vaisman A, Kuban W, McDonald JP, Karata K, Yang W, Goodman MF, Woodgate R. Critical amino acids in *Escherichia coli* UmuC responsible for sugar discrimination and base-substitution fidelity. *Nucleic Acids Res*. 2012; 40:6144–6157. [PubMed: 22422840]
23. Donigan KA, McLenigan MP, Yang W, Goodman MF, Woodgate R. The steric gate of DNA polymerase iota regulates ribonucleotide incorporation and deoxyribonucleotide fidelity. *J Biol Chem*. 2014; 289:9136–9145. [PubMed: 24532793]
24. Niimi N, Sassa A, Katafuchi A, Gruz P, Fujimoto H, Bonala RR, Johnson F, Ohta T, Nohmi T. The steric gate amino acid tyrosine 112 is required for efficient mismatched-primer extension by human DNA polymerase kappa. *Biochemistry*. 2009; 48:4239–4246. [PubMed: 19341290]
25. Ruiz JF, Juarez R, Garcia-Diaz M, Terrados G, Picher AJ, Gonzalez-Barrera S, Fernandez de Henestrosa AR, Blanco L. Lack of sugar discrimination by human Pol mu requires a single glycine residue. *Nucleic Acids Res*. 2003; 31:4441–4449. [PubMed: 12888504]

26. Kirouac KN, Suo Z, Ling H. Structural mechanism of ribonucleotide discrimination by a Y-family DNA polymerase. *J Mol Biol.* 2011; 407:382–390. [PubMed: 21295588]
27. Joyce CM, Potapova O, Delucia AM, Huang X, Basu VP, Grindley ND. Fingers-closing and other rapid conformational changes in DNA polymerase I (Klenow fragment) and their role in nucleotide selectivity. *Biochemistry.* 2008; 47:6103–6116. [PubMed: 18473481]
28. Wang W, Wu EY, Hellinga HW, Beese LS. Structural factors that determine selectivity of a high fidelity DNA polymerase for deoxy-, dideoxy-, and ribonucleotides. *J Biol Chem.* 2012; 287:28215–28226. [PubMed: 22648417]
29. Shurtleff BW, Ollivierre JN, Tehrani M, Walker GC, Beuning PJ. Steric Gate Variants of UmuC Confer UV Hypersensitivity on *Escherichia coli*. *J Bacteriol.* 2009; 191:4815–4823. [PubMed: 19482923]
30. Benson RW, Norton MD, Lin I, Du Comb WS, Godoy VG. An active site aromatic triad in *Escherichia coli* DNA Pol IV coordinates cell survival and mutagenesis in different DNA damaging agents. *PLoS One.* 2011; 6:e19944. [PubMed: 21614131]
31. Sharma A, Kottur J, Narayanan N, Nair DT. A strategically located serine residue is critical for the mutator activity of DNA polymerase IV from *Escherichia coli*. *Nucleic Acids Res.* 2013; 41:5104–5114. [PubMed: 23525461]
32. Zhou BL, Pata JD, Steitz TA. Crystal structure of a DinB lesion bypass DNA polymerase catalytic fragment reveals a classic polymerase catalytic domain. *Mol Cell.* 2001; 8:427–437. [PubMed: 11545744]
33. Ummat A, Silverstein TD, Jain R, Buku A, Johnson RE, Prakash L, Prakash S, Aggarwal AK. Human DNA polymerase eta is pre-aligned for dNTP binding and catalysis. *J Mol Biol.* 2012; 415:627–634. [PubMed: 22154937]
34. Katta V, Chait BT. Conformational changes in proteins probed by hydrogen-exchange electrospray-ionization mass spectrometry. *Rapid Commun Mass Spectrom.* 1991; 5:214–217. [PubMed: 1666528]
35. Zhang Z, Smith DL. Determination of amide hydrogen exchange by mass spectrometry: a new tool for protein structure elucidation. *Protein Sci.* 1993; 2:522–531. [PubMed: 8390883]
36. Konermann L, Vahidi S, Sowole MA. Mass spectrometry methods for studying structure and dynamics of biological macromolecules. *Anal Chem.* 2014; 86:213–232. [PubMed: 24304427]
37. Englander SW, Downer NW, Teitelbaum H. Hydrogen exchange. *Annu Rev Biochem.* 1972; 41:903–924. [PubMed: 4563445]
38. Wales TE, Engen JR. Hydrogen exchange mass spectrometry for the analysis of protein dynamics. *Mass Spectrom Rev.* 2006; 25:158–170. [PubMed: 16208684]
39. Beuning PJ, Simon SM, Godoy VG, Jarosz DF, Walker GC. Characterization of *Escherichia coli* translesion synthesis polymerases and their accessory factors. *Methods Enzymol.* 2006; 408:318–340. [PubMed: 16793378]
40. Irimia A, Eoff RL, Guengerich FP, Egli M. Structural and functional elucidation of the mechanism promoting error-prone synthesis by human DNA polymerase kappa opposite the 7,8-dihydro-8-oxo-2'-deoxyguanosine adduct. *J Biol Chem.* 2009; 284:22467–22480. [PubMed: 19542228]
41. Sambrook, J.; Fritsch, EF.; Maniatis, T. *Molecular Cloning--A laboratory manual.* Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 1989.
42. Wales TE, Fadgen KE, Gerhardt GC, Engen JR. High-speed and high-resolution UPLC separation at zero degrees Celsius. *Anal Chem.* 2008; 80:6815–6820. [PubMed: 18672890]
43. Wang L, Pan H, Smith DL. Hydrogen exchange-mass spectrometry: optimization of digestion conditions. *Mol Cell Proteomics.* 2002; 1:132–138. [PubMed: 12096131]
44. Geromanos SJ, Vissers JP, Silva JC, Dorschel CA, Li GZ, Gorenstein MV, Bateman RH, Langridge JI. The detection, correlation, and comparison of peptide precursor and product ions from data independent LC-MS with data dependant LC-MS/MS. *Proteomics.* 2009; 9:1683–1695. [PubMed: 19294628]
45. Houde D, Berkowitz SA, Engen JR. The utility of hydrogen/deuterium exchange mass spectrometry in biopharmaceutical comparability studies. *J Pharm Sci.* 2011; 100:2071–2086. [PubMed: 21491437]

46. Walsh JM, Bouamaied I, Brown T, Wilhelmsson LM, Beuning PJ. Discrimination against the cytosine analog tC by *Escherichia coli* DNA polymerase IV DinB. *J Mol Biol.* 2011; 409:89–100. [PubMed: 21477595]
47. Fang J, Nevin P, Kairys V, Venclovas C, Engen JR, Beuning PJ. Conformational analysis of processivity clamps in solution demonstrates that tertiary structure does not correlate with protein dynamics. *Structure.* 2014; 22:572–581. [PubMed: 24613485]
48. Lone S, Townson SA, Uljon SN, Johnson RE, Brahma A, Nair DT, Prakash S, Prakash L, Aggarwal AK. Human DNA polymerase kappa encircles DNA: implications for mismatch extension and lesion bypass. *Mol Cell.* 2007; 25:601–614. [PubMed: 17317631]
49. Santoso Y, Joyce CM, Potapova O, Le Reste L, Hohlbein J, Torella JP, Grindley ND, Kapanidis AN. Conformational transitions in DNA polymerase I revealed by single-molecule FRET. *Proc Natl Acad Sci U S A.* 2010; 107:715–720. [PubMed: 20080740]
50. Hohlbein J, Aigrain L, Craggs TD, Bermek O, Potapova O, Shoolizadeh P, Grindley ND, Joyce CM, Kapanidis AN. Conformational landscapes of DNA polymerase I and mutator derivatives establish fidelity checkpoints for nucleotide insertion. *Nat Commun.* 2013; 4:2131. [PubMed: 23831915]
51. Jarosz DF, Cohen SE, Delaney JC, Essigmann JM, Walker GC. A DinB variant reveals diverse physiological consequences of incomplete TLS extension by a Y-family DNA polymerase. *Proc Natl Acad Sci U S A.* 2009; 106:21137–21142. [PubMed: 19948952]

Highlights

- DinB and pol κ probed by hydrogen/deuterium exchange (HDX)-mass spectrometry
- DinB and pol κ had reduced HDX in ternary complexes with dNTP relative to rNTP
- Mutation of steric gate resulted in ternary complexes with sugar-independent HDX
- The steric gate residue is crucial for dNTP-induced conformational change
- Discrimination against rNTP occurs in a closed polymerase conformation

FIGURE 1. Hydrogen exchange of WT DNA polymerases DinB and pol κ and steric gate variants in the absence of substrates

(a) The steric gate model: A bulky residue prevents rNTP incorporation due to steric clash with 2'-OH of ribose. (b) Peptide-level HDX data were mapped together with atomic-level structural information to visualize the intrinsic local structural dynamics of the free polymerases in solution. For selected time points shown, the percent deuterium uptake for each peptide is displayed as a color gradient on the ternary complex crystal structure of DinB (PDB: 4IRC) and pol κ (PDB: 2OH2). DNA and nucleotides are not shown. HDX data are not corrected for back-exchange and the theoretical maximum number of deuterated

amides in each peptide equals its number of residues after subtracting one for the N-terminus and one for each proline residue. (c) Differences in deuterium uptake between WT and steric gate variants of DinB (left) and pol κ (right) displayed as a color gradient for each peptide at the indicated time points. The deuterium uptake for the steric gate variant was subtracted from that of the respective WT. Differences >0.6 Da are significant at 98% confidence. (d) Deuterium uptake for DinB residues 77–88 (left) and the corresponding pol κ residues 165–180 indicating faster HDX in the steric gate variants. (e) DinB palm domain residues 77–88 (red), F13 (magenta), Y79 (cyan), the dNTP (green), and DNA (yellow) highlighted on the structure of DinB (PDB: 4IRC). Magnesium ions are shown as spheres. (f) Deuterium uptake differences >1 Da between WT pol κ and Y112A from (c, right) mapped on the ternary complex crystal structure of pol κ (PDB: 2OH2). (2 columns, color web and print)

FIGURE 2. Substrate-dependent thermal stability of DinB and pol κ

DinB (top) and pol κ (bottom) variants were incubated with substrates and heated in the presence of Sypro orange. The protein variant, templating base, and incoming nucleotide are indicated in the legend. (1 column, grayscale web and print)

FIGURE 3. Reduced ribonucleotide discrimination by steric gate variants

Primer extension products of DinB (a) and pol κ (b) variants on undamaged DNA in the presence of dNTPs or rNTPs. Reactions were quenched after 0, 5, 15, and 60 min and analyzed by 16% denaturing polyacrylamide gel electrophoresis and subsequent phosphorimaging. (1 column, grayscale web and print)

FIGURE 4. DinB is protected from HDX by dNTP but not rNTP

(a) Differences in deuterium uptake between the dCTP- and rCTP-bound states of DinB displayed as a color gradient for each peptide at the indicated time points. In each case (WT and F13A), differences were calculated by subtracting the relative deuterium uptake for DinB-DNA-dCTP from that of DinB-DNA-rCTP. The templating base was G and a dideoxy-terminated primer was used to avoid nucleotide incorporation. White indicates that no data were obtained. (b) Regions of WT DinB showing HDX differences between the dCTP- and the rCTP-bound state >1 Da at any time point displayed as a color gradient on the ternary complex crystal structure (PDB: 4IRC). In this image, brown indicates regions for which no data were obtained. The incoming nucleotide is colored green and F13 is colored magenta. (c) Specific residues highlighted in different colors on the DinB structure. F13 (magenta), Y79 (yellow), and the nucleotide (green) are shown as sticks. The red star is a point of reference. (2 columns, color web and print)

FIGURE 5. Pol κ is protected from HDX by dNTP but not rNTP

(a) Differences in deuterium uptake between the dCTP- and rCTP-bound states of pol κ displayed as a color gradient for each peptide at the indicated time points. In each case (WT and Y112A), differences were calculated by subtracting the relative deuterium uptake for pol κ -DNA-dCTP from that of pol κ -DNA-rCTP. The templating base was G and a dideoxy-terminated primer was used to avoid nucleotide incorporation. White indicates that no data were obtained. (b) Regions of WT pol κ showing HDX differences between the dCTP- and the rCTP-bound state >1 Da at any time point displayed as a color gradient on the ternary complex crystal structure (PDB: 2OH2). In this image, brown indicates regions for which no data were obtained. The incoming nucleotide is colored green and Y112 is colored magenta. (c) Specific residues highlighted in different colors on the pol κ structure. Y112 (magenta), Y174 (yellow), and the nucleotide (green) are shown as sticks. (2 columns, color web and print)

FIGURE 6. Steric gate variants do not favor the protected state

(a) Differences in deuterium uptake between the rCTP-bound state of WT DinB and the rCTP- (left) or dCTP-bound (right) state of DinB(F13A) displayed as a color gradient for each peptide at the indicated time points. The relative deuterium uptake for DinB-DNA-rCTP was subtracted from that of F13A-DNA-rCTP (left) or F13A-DNA-dCTP (right). White indicates that no data were obtained. The templating base was G and a dideoxy-terminated primer was used to avoid nucleotide incorporation. (b) Deuterium uptake for the indicated DinB residues. (c) Differences in deuterium uptake between the rCTP-bound state of WT pol κ and the rCTP- (left) or dCTP-bound (right) state of pol κ (Y112A). The relative deuterium uptake for pol κ -DNA-rCTP was subtracted from that of Y112A-DNA-rCTP (left) or Y112A-DNA-dCTP (right). (d) Deuterium uptake for the indicated pol κ residues. (2 columns, color web and print)