The Properties of Steric Gate Mutants Reveal Different Constraints within the Active Sites of Y-family and A-family DNA Polymerases*

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Y-family (lesion-bypass) DNA polymerases show the same overall structural features seen in other members of the polymerase superfamily, yet their active sites are more open, with fewer contacts to the DNA and nucleotide substrates. This raises the question of whether analogous active-site side chains play equivalent roles in the bypass polymerases and their classical DNA polymerase counterparts. In Klenow fragment, an A-family DNA polymerase, the steric gate side chain (Glu710) not only prevents ribonucleotide incorporation but also plays an important role in discrimination against purine-pyrimidine mismatches. In this work we show that the steric gate (Phe12) of the Y-family polymerase Dbh plays a very minor role in fidelity, despite its analogous role in sugar selection. Using ribonucleotide discrimination to report on the positioning of a mispaired dNTP, we found that the pyrimidine of a Pu-dPyTP nascent mispair occupies a similar position to that of a correctly paired dNTP in the Dbh active site, whereas in Klenow fragment the mispaired dNTP sits higher in the active site pocket. If purine-pyrimidine mismatches adopt the expected wobble geometry, the difference between the two polymerases can be attributed to the binding of the templating base, with the looser binding site of Dbh permitting a variety of template conformations with only minimal adjustment at the incoming dNTP. In Klenow fragment the templating base is more rigidly held, so that changes in base pair geometry would affect the dNTP position, allowing the Glu710 side chain to serve as a sensor of nascent mismatches.

Dbh (DinB homologue) is a Y-family DNA polymerase from the thermophilic archaeabacterium Sulfolobus acidocaldarius. Y-family polymerases comprise a diverse group of low fidelity enzymes that are specialized for a mode of DNA synthesis that involves bypass of DNA damage or helix distortions (1). The replication fidelity of Dbh (and DinB polymerases from other organisms) is ~10⁻² to 10⁻³ lower than that of the “classical” replicative and repair polymerases exemplified by families A and B (2–5).

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2 The abbreviations used are: Dbh, DinB homologue; dNTP, deoxyribonucleoside triphosphate; rNTP, ribonucleoside triphosphate; Pu, purine; Py, pyrimidine.
entry of nascent mispairs bound to Dbh and Klenow fragment. Our findings suggest there are significant differences in the way these two DNA polymerases accommodate nascent mispairs.

**EXPERIMENTAL PROCEDURES**

**Materials**—DNA oligonucleotides were synthesized by the Keck Biotechnology Resource Laboratory at Yale Medical School. Duplex DNA substrates for kinetic experiments, 5‘-labeled on the primer strand, were prepared by annealing appropriate template and primer strands. Ultrapure dNTPs and rNTPs were purchased from Amersham Biosciences, except for rTTP, which was from TriLink Biotechnologies. Purification of Dbh (wild-type and F12A) and 3‘-5’ exonuclease-deficient Klenow fragment (D424A) proteins followed our standard procedures (19, 27). Klenow fragment derivatives relevant to this work all carry the D424A mutation but are described here simply by the genotype of their polymerase domain.

**Kinetic Measurements**—All kinetic measurements were carried out under single-turnover conditions, using a rapid quench-flow instrument (KinTek Corp., model RQF-3) for reactions that were too fast to stop by manual quenching. For Klenow fragment, reactions were carried out at 22 °C as described previously (26). For Dbh, measurements were made at 37 °C, based on our published methods (2, 19) with the following modifications. Reactions were initiated by mixing equal volumes of a dNTP or rNTP solution with a preincubated Dbh-DNA solution. In each reaction extra MgCl₂ (equimolar with the dNTP or rNTP) was provided in addition to the 10 mM MgCl₂ already present in the Dbh reaction buffer. All reaction products were analyzed as described previously (2).

**RESULTS**

**Misinsertion Kinetics for Wild-type and F12A Dbh**—To investigate whether the steric gate residue (Phe12) of Dbh plays a role in polymerase fidelity that is analogous to that of the steric gate residue (Glu710) in Klenow fragment, we measured the discrimination against T-dGTP and G-dTTP mispairs by wild-type and F12A Dbh (Table 1). The rate of incorporation (kₚₒₒ) of the correct and incorrect nucleotide was measured at a series of nucleotide concentrations under single-turnover conditions at 37 °C. From plots of the first-order rate constant (kₜₒₒ) as a function of nucleotide concentration, we determined the maximum rate of nucleotide incorporation (kₚₒ) and nucleotide binding affinity (Kᵣ) for the Dbh-DNA complex (Fig. 1). Selection against formation of T-dGTP or G-dTTP mispairs was calculated by dividing the efficiency (kₚₒₒ/Kᵣ) for correct nucleotide addition by the efficiency for incorrect nucleotide addition.

From the pre-steady-state kinetic constants for correct base pair and mispair formation by wild-type and F12A Dbh (Table 1), the discrimination by wild-type Dbh against wobble mismatches was calculated to be ~10⁻³, with 5-fold greater discrimination against G-dTTP mispairs than T-dGTP mispairs. For both mismatches, the differences in dNTP binding between the correct and incorrect nucleotide were small (3–7-fold) compared with the changes in rate of nucleotide addition (300–540-fold), showing that transition state interactions are the main source of selectivity against these mismatches. Mutation of the steric gate residue, Phe12, to alanine had very little effect on the level of discrimination against either the T-dGTP or G-dTTP mismatch. In each case, the decrease in incorporation efficiency due to the F12A mutation was similar in magnitude for the correct base pair and the corresponding mispair, resulting in no overall change in fidelity. For both the template-T and template-G substrate, the F12A mutation caused a modest decrease (3–5-fold) in binding affinity for the correct dNTP but did not affect binding of the incorrect nucleotide. The F12A mutation also caused a moderate decrease (3–20-fold) in the rate of dNTP addition. The rate decrease was larger for misincorpora-

TABLE 1

| Protein      | Correct dNTP | Incorrect dNTP |
|--------------|--------------|----------------|
|              | Kᵣ (mM)     | kₚₒₒ (s⁻¹)   | kₚₒₒ/Kᵣ |
| G-dCTP       | 0.89 ± 0.06  | 7.0 ± 0.2     | 7.9 × 10⁶ |
|              | 4.4 ± 0.5    | 2.4 ± 0.2     | 530      |
| T-dATP       | 2.2 ± 1.3    | 8.9 ± 1.1     | 4.0 × 10⁷ |
|              | 7.2 ± 0.01   | 0.55 ± 0.18   | 76       |
|              | 6.4 ± 3.0    | (1.3 ± 0.03) × 10⁻² | 2.0   |
|              | 5.4 ± 0.4    | (9.5 ± 1.4) × 10⁻⁴ | 0.17   |
|              | 6.2 ± 0.8    | (3.0 ± 0.4) × 10⁻² | 4.7    |
|              | 8.2 ± 3.6    | (1.3 ± 0.3) × 10⁻³ | 0.16   |

* Calculated as (kₚₒₒ/Kᵣ)correct/(kₚₒₒ/Kᵣ)incorrect.

**FIGURE 1. Kinetics of dGTP-T misincorporation by F12A Dbh.** The first-order rate constants (kₚₒₒ) for dGTP addition to the DNA duplex, whose sequence is given in Table 1, were plotted against dGTP concentration. The solid line represents the best fit of the data to a hyperbolic equation, giving a dissociation constant (Kᵣ) of 5.6 mM for binding of dGTP to the complex of F12A Dbh with primer/template and a maximum incorporation rate (kₚₒₒ) of 1.0 × 10⁻³ s⁻¹.
The decrease in dTTP/rUTP discrimination was entirely due to the opposing effect of F12A on nucleotide binding, this accounts for the negligible effect of this mutation on fidelity.

Use of rNTP Discrimination to Report on Positioning of Wobble Mispairs—We used the level of discrimination against rNTP incorporation to report on the positioning of nascent mispairs at the polymerase active sites of Dbh and Klenow fragment. The selectivity for dNTPs over rNTPs depends on the positioning of the 2'-OH on the sugar of the incoming nucleotide relative to the steric gate side chain. If an incoming incorrect nucleotide occupies a similar position to that of the corresponding correct nucleotide, then both incorporation reactions should show similar selectivity for the nucleotide sugar. An increase in dNTP/rNTP selectivity in a misinsertion reaction would imply that the incorrect incoming nucleotide is closer to the steric gate side chain than a correctly paired nucleotide; the opposite conclusion would be inferred from a decrease in selectivity.

We assessed the dNTP/rNTP selectivity of wild-type Dbh by comparing the rates of polymerase-catalyzed incorporation at a single concentration of 10 mM nucleotide (Table 2). We adopted this approach, rather than determining individual $k_{pol}$ and $K_d$ values, because our previous studies (2, 19) and this work (Table 1) indicated that changes in the reaction substrates primarily affect the rate of nucleotide addition and have little influence on the rather weak nucleotide binding affinity of Dbh. The rate constants are reported as $k_{obs}$, not $k_{pol}$, values because the nucleotide binding of Dbh is not saturated at 10 ms (see Fig. 1). Comparison of the rate constants at 10 and 2 mM nucleotide, for each reaction, supported our expectation that there would be no dramatic changes in nucleotide binding affinity in this series of experiments. For the majority of the reactions, the rate at 2 mM nucleotide was about 40% of that at 10 mM, consistent with a $K_d$ of around 6 mM. For correct dNTP incorporation by wild-type Dbh, the rate at 2 mM was 60 to 70% of that at 10 mM, indicating a $K_d$ of 1–2 mM.

Discrimination by wild-type Dbh against rNTPs in correct Watson-Crick pairings was ~20,000-fold, except for rCTP where the discrimination was 10-fold lower5 (Table 2). For an incoming pyrimidine mispaired opposite a template purine, the rNTP discrimination was slightly lower than for the corresponding correct base pair. When the incoming nucleotide was a purine, rNTP discrimination was essentially the same regardless of whether or not the nucleotide was correctly paired with the templating base. As expected, the absence of the steric gate side chain, in the F12A Dbh mutant, resulted in very little discrimination against rNTPs (less than 10-fold) in all reactions tested (data not shown).

Klenow fragment, with a more constrained nucleotide binding pocket than Dbh, normally binds a correct nucleotide with ~100-fold higher affinity than does Dbh. Therefore the consequences of mispair geometry (reported by rNTP discrimination) at the active site of Klenow fragment may be seen both at the level of nucleotide binding and nucleotide incorporation rate. We compared the single-turnover kinetics for rGTP and rUTP incorporation in correct and wobble mispairs by Klenow fragment at a series of nucleotide concentrations so as to determine $k_{pol}$ and $K_d$ (Table 3). As with Dbh, selection against rNTP addition in a base pair with an incorrect incoming purine (T-d/rGTP) was very similar to dGTP/rGTP discrimination in a correct base pair. However, with the opposite arrangement, an incoming pyrimidine mispaired with a template purine, ribonucleotide discrimination was substantially reduced, with the discrimination between dTTP and rUTP ~300-fold lower when mispaired with G than when correctly paired with A. The decrease in dTTP/rUTP discrimination was entirely due to

| Correct d/rNTP discrimination | Incorrect d/rNTP discrimination |
|-------------------------------|---------------------------------|
| dNTP $k_{obs}$ (s$^{-1}$) | rNTP $k_{obs}$ (s$^{-1}$) | Selectivitya | dNTP $k_{obs}$ (s$^{-1}$) | rNTP $k_{obs}$ (s$^{-1}$) | Selectivitya |
| A-dTTP | A-rUTP | dT/rU | (7.8 ± 1.2) × 10$^{-3}$ | (1.4 ± 0.9) × 10$^{-6}$ | 5.7 × 10$^2$ |
| G-dCTP | G-rCTP | dC/rC | (1.1 ± 0.2) × 10$^{-6}$ | 6.9 × 10$^3$ |
| C-dGTP | C-rGTP | dG/rG | (2.4 ± 0.4) × 10$^{-5}$ | (3.5 ± 1.8) × 10$^{-6}$ | 670 |
| T-dATP | T-rATP | dA/rA | (1.8 ± 0.4) × 10$^{-2}$ | (6.0 ± 1.8) × 10$^{-7}$ | 3.1 × 10$^4$ |

a Calculated as $(k_{obs})_{dNTP}/(k_{obs})_{rNTP}$

5 The different behavior of rCTP is interesting in view of the preferred incorporation of dCTP by Dbh, reported previously (2).
nucleotide is correct or mismatched (Table 1). The only observation that hints at a role for Phe12 in mismatch recognition is a slight trend in the $K_d$ (dNTP) values for both mismatches tested. Binding of the correctly paired dNTP by wild-type Dbh is tighter (by 3–7-fold) than binding by the F12A mutant or binding by either protein of an incoming mismatched dNTP, perhaps indicating a weak interaction that is compromised either in the absence of Phe12 or in a mismatched nascent base pair.

The ribonucleotide discrimination data, which report on the position of the incoming nucleotide, provide a plausible explanation for the different roles of the steric gate side chain in Klenow fragment and Dbh (Tables 2 and 3). In Dbh the dNTP/rNTP discrimination is similar regardless of whether or not the incoming nucleotide is correctly paired with the template base. This indicates that the position of the sugar of an incoming dNTP relative to Phe12 is approximately the same in either situation and therefore that the Phe12 side chain would not be an effective sensor of nascent mispairs. Conversely, the ribonucleotide data for Klenow fragment imply that an incoming dTTP is further from the steric gate side chain when mispaired opposite G than when correctly paired opposite A. The favorable interaction of the nucleotide with Glu710 is therefore less in the mispair and Glu710 serves as a discriminator against Pu-dPyTP errors. This discrimination is lost in the E710A mutant, accounting for its mutator phenotype. Two possible reasons underlying the different properties of steric gate mutants of Klenow fragment and Dbh, one structural and the other mechanistic, are examined below.

**Active Site Structure**—The structural explanation assumes that the misincorporation pathway proceeds via a ternary complex that resembles the ternary complexes seen with correctly paired incoming dNTPs, with appropriate adjustments to accommodate the mispair. In Klenow fragment, the ribonucleotide discrimination data imply that an incoming dTTP sits higher in the active site binding pocket when mispaired with G

### DISCUSSION

**Misinsertion Fidelity of Dbh**—The pre-steady-state kinetic data for correct and incorrect Pu-Py base pairs show that Dbh has a lower misinsertion fidelity than Klenow fragment, as expected from our earlier measurements of forward mutation frequencies (2). Relative to wild-type Klenow fragment, mismatch discrimination by wild-type Dbh is 25-fold less for G-dTTP and 5-fold less for T-dGTP mismatches (Table 1 and Ref. 26). Like Klenow fragment, Dbh shows greater discrimination against G-dTTP mispairs than T-dGTP mispairs (26). For both mispairs in this study, discrimination by Dbh derives largely from differences in reaction rate, consistent with other kinetic studies of Y-family polymerases (4, 28, 29).

Our results indicate that the steric gate residue, Phe12, of Dbh plays very little role in purine-pyrimidine mismatch fidelity, in contrast to the steric gate residue, Glu710, of Klenow fragment. The E710A Klenow fragment is a mutator for Pu-dPyTP mismatches, and the ~100-fold loss in selectivity responsible for this phenotype results from the complete loss of discrimination in dNTP binding and ~10-fold lower selectivity in $k_{pol}$ relative to wild-type Klenow fragment (26). With the opposite mismatch, Py-dPuTP, E710A shows substantial changes in the individual $k_{pol}$ and $K_d$ values compared with wild-type Klenow fragment. Nevertheless, E710A is not a mutator for Py-dPuTP errors because the $k_{pol}$ and $K_d$ changes are in opposite directions, reflecting tighter binding of the mispaired purine but a severely compromised catalytic rate. In contrast to E710A Klenow fragment, the F12A mutation in Dbh results in similar decreases in overall activity, regardless of whether the incoming

### Changes in ground-state interactions. Because discrimination by Klenow fragment against rUTP has been observed to be particularly high (Table 3 and Ref. 20), we repeated the measurements with rUTP. The results were very similar to those obtained with rUTP, ruling out any unusual effects due to the thymine 5-methyl group.

### Table 3

| Correct d/rNTP discrimination | Incorrect d/rNTP discrimination |
|-------------------------------|---------------------------------|
| **Substrates**                | **$K_d$ ($\mu$M) $k_{pol}$ (s$^{-1}$) $k_{pol}/K_d$ (M$^{-1}$s$^{-1}$) Selectivity** |
| dTTP                         | 13 $1.3 \times 10^7$            |
| rUTP                         | 960 $3.6 \times 10^{-2}$        |
| rTTP                         | ND $3.3 \times 10^5$            |
| G-dT/rUTP                    | 750 $3.4 \times 10^{-2}$        |
| rUTP                         | 200 $7.4 \times 10^{-6}$        |
| rTTP                         | 340 $2.4 \times 10^{-5}$        |
| T-d/rGTP                     | 48 $3.4 \times 10^3$            |
| rUTP                         | 110 $2.0 \times 10^{-5}$        |

*Where X was A or G, as appropriate.

*Where X was C or T, as appropriate.

*Calculated as $(k_{pol}/K_d)_{Klenow}/(k_{pol}/K_d)_{Dbh}.*
Purine-Pyrimidine Mismatch Discrimination

A

major groove

G-C

G-T

minor groove

B

C

FIGURE 2. Hypothetical structures for bound mispairs. A, comparison of a correct G-C pair (gold) and a wobble G-T pair (green). Coordinates for the two base pairs were taken from the structure file bdl009 (30) and superimposed by overlapping corresponding C1′ positions. B, the active site pocket of an A-family DNA polymerase, KlenTaq, taken from Protein Data Bank file 3KTQ (11). The protein is shown as a surface, with most of the thumb subdomain removed because it would otherwise obstruct the view into the active site. The nascent base pair has a template G (blue) paired with dCTP (silver). When a wobble G-T mispair (from bdl009) is introduced so that the G base is exactly superimposed on the template G of the nascent base pair, the mispaired T base (semitransparent green) is displaced toward the major groove relative to the correct dCTP. The positions of the following KlenTaq side chains, with their Klenow fragment homologues in parentheses, are indicated: Glu615 (Glu710), yellow; Phe667 (Phe762), cyan; Tyr761 (Tyr766), magenta. C, a correctly paired A-dTTP in the active site pocket of Dpo4, taken from Protein Data Bank file 1500 (32, 36). The protein is shown as a surface, with the thumb and little finger subdomains omitted so as not to obstruct the view of the active site. The templating A is blue and the incoming dTTP silver. When a wobble G-T mispair (from bdl009) is introduced so that the T base is exactly superimposed on the base of the incoming dTTP, the mispaired template G base (semitransparent green) is displaced toward the minor groove relative to the correct template A. The positions of the following Dpo4 side chains, with the equivalent Dbh side chains in parentheses, are indicated: Tyr14 (Phe13), yellow; Val32 (Val32), magenta; Ala44 (Ala44), cyan; Met76 (Met76), blue. Throughout this figure, the base pairs are oriented consistently so that the major groove is toward the top and the minor groove toward the bottom. The positions of mispairs are for illustration only and make no allowance for adjustments to optimize the fit with the protein, as would be necessary to alleviate a slight steric clash between the mispaired template G and Met76 in the Dbh binding pocket. This figure was made using PyMOL (DeLano Scientific).
ily polymerases do not have a large side chain in the position equivalent to the invariant tyrosine of A-family DNA polymerases (Tyr<sup>673</sup> of Klentaq, and Tyr<sup>266</sup> of Klenow fragment), which, in the ternary complex, not only forms the floor of the binding pocket on the template side (Fig. 2B) but also contributes to the rigidity of the entire binding pocket with a hydrogen bond from the tyrosine hydroxyl to the steric gate carboxylate (13). The ternary complex of Dpo4, a close homologue of Dbh, has a much smaller side chain (Val<sup>52</sup>) in an analogous position on the minor groove side of the templating base (Fig. 2C). Moreover, this part of the binding pocket resembles a narrow ledge more than a floor; in contrast to the situation in Klenow fragment, it does not extend to the surface of the primer terminal base pair to form a continuous contact surface for the nascent base pair. It therefore seems reasonable that some repacking of the small side chains that make up the Dpo4 or Dbh binding pocket could take place in order to accommodate a variety of template base positions. As illustrated in Fig. 2C, this would be required in a Pu-dPyTP wobble mismatch if the incoming pyrimidine occupied a position identical to that in a correct base pair. Alternatively, the adjustments to accommodate wobble geometry could be shared between template and dNTP sides of the binding site, as appears to be the case in a comparison of T-dATP and T-dGTP base pairs bound to Dpo4 (32).

In summary, we suggest that the flexibility on the template side of the Dbh active site allows for a consistent location of the incoming dNTP regardless of whether or not it is correctly paired with its templating partner. Contact of the dNTP sugar with the Phe<sup>13</sup> steric gate side chain is maintained in all circumstances with the result that Dbh shows stringent discrimination against ribonucleotides but does not use the steric gate side chain as a discriminator against nascent mispairs.

**Reaction Pathway**—The alternative mechanistic explanation takes account of the possibility of distinct reaction pathways for polymerase addition depending on whether the incoming dNTP is correct or incorrect (33). In Dbh, the conformation of the active site binding pocket does not change significantly on binding substrates, suggesting that a nascent mispair may be accommodated, with minor adjustments, as described in the previous section. By contrast, in an A-family DNA polymerase such as Klenow fragment, part of the fingers subdomain moves so as to form the closed complex in response to the binding of a correctly matched dNTP (11–13, 16). It is this movement that forms the snug binding pocket for the nascent base pair and brings the dNTP sugar close to the steric gate side chain, Glu<sup>710</sup> of Klenow fragment. If the fingers-closing transition were absent or altered in the misincorporation pathway, mispairs would interact to a lesser extent with Glu<sup>710</sup>, accounting for the changes in ribonucleotide discrimination and for the ability of Glu<sup>710</sup> to serve as a discriminator against mispairs. Assessment of the plausibility of this mechanistic explanation will require a fuller understanding of the differences, if any, between the pathways for correct and incorrect dNTP addition, although it is worth noting that fluorescence experiments suggest that significant differences may exist (34, 35).  

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4 C. M. Joyce, X. Huang, O. Potapova, and N. D. F. Grindley, unpublished observations.

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