Importance of Hydrogen Bonding for Efficiency and Specificity of the Human Mitochondrial DNA Polymerase*

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To assess the contribution to discrimination afforded by base pair hydrogen bonding during DNA replication by the human mitochondrial DNA polymerase, we examined nucleoside mimics lacking hydrogen bond forming capability but retaining the overall steric shape of the natural nucleotide. We employed oligonucleotide templates containing either a deoxyadenosine shape mimic (dQ) or a deoxythymidine shape mimic (dF). Additionally, the nucleoside triphosphate analogs difluorotoluene deoxynucleoside triphosphate, 9-methyl-1-H-imidazo[(4,5)-b]pyridine deoxyribose triphosphate, and 4-methylbenzimidazole deoxyribose triphosphate (dZTP; another dATP shape mimic) were assayed. We used pre-steady state methods to determine the kinetic parameters governing nucleotide incorporation, $k_{pol}$ and $K_d$. In general, the loss of hydrogen bonding potential led to 2–3 kcal/mol reduction in ground state binding free energy, whereas effects on the maximum rate of polymerization were quite variable, ranging from negligible (dATP:dF) to nearly 4 kcal/mol (dZTP:dT). Although we observed only a 46-fold reduction in discrimination when dF was present in the template, there was a complete elimination of discrimination when dQ was present in the template. Our data with dF indicate that hydrogen bonding contributes 2.2 kcal/mol toward the efficiency of incorporation, whereas data with dQ (which may overestimate the effect due to poor steric mimicry) suggest a contribution of up to 6.8 kcal/mol. Taken together, the data suggest that sterics are necessary but not sufficient to achieve optimal efficiency and fidelity for DNA polymerase. Base pair hydrogen bonding contributes at least a third of the energy underlying nucleoside incorporation efficiency and specificity.

Several factors can contribute to DNA replication fidelity, including base pair hydrogen bonding, base pair geometry, and base stacking, and each of these factors is affected by the environment at the DNA polymerase active site (1–3). DNA replication follows a minimal pathway illustrated in Fig. 1, where $K_{dNTP}$ represents the apparent dissociation constant for ground state nucleotide binding, and $k_{pol}$ defines the rate of polymerization at the active site (2). After incorporation of one nucleotide, the enzyme-DNA complex can then either bind another nucleotide to continue processive synthesis, the DNA can dissociate at a rate $k_{off}$, or the DNA can migrate to the exonuclease site and undergo hydrolysis of the 3'-terminal base of the primer at rate $k_{exonuc}$. In this report we quantify specificity by measuring the concentration dependence of single nucleotide incorporation events to define the specificity constant, equal to $k_{pol}/K_d$. Although $K_d$ may be limited by either chemistry or a nucleotide-induced conformational change, these measurements accurately define polymerase specificity and efficiency (2, 4).

Over a range of DNA polymerases with widely varying fidelity, the ground state binding ($K_d$) contributes a factor of $\approx 200–300$ toward the net selectivity, whereas $k_{pol}$ contributes between 50- and 2000-fold to achieve variable fidelity for different polymerases (5–7). The ground state binding contribution to fidelity is nearly identical to that which is calculated from solution studies of base pair hydrogen bonding, and therefore, it has been suggested that ground state nucleotide binding at the polymerase active site is dominated by formation of the base pair hydrogen bonds with the templating base, whereas the conformational change and/or chemistry steps are dependent upon optimal base pair geometry (2) that may be enforced sterically (8, 9). Accordingly, lower fidelity enzymes with a more open active site do not enforce base pair geometry to the same extent as a higher fidelity enzyme.

To resolve the contributions of shape and base pair hydrogen bonding in DNA polymerase fidelity, nucleoside analogs were developed that mimic the overall shape but lack hydrogen bonding capability (10–14). Difluorotoluene deoxynucleoside (dF) is a nonpolar analog of deoxythymidine (10) in which the carbonyl oxygen atoms of dT are replaced by fluorine while retaining nearly the exact shape of dT. Difluorotoluene has been shown to lack base pair hydrogen bonding ability with adenine in either DNA or RNA and even in chloroform, a solvent that strongly favors hydrogen bond formation (11, 15, 16). Additionally, when paired opposite a dA in a DNA duplex, dF destabilizes the duplex and shows no pairing selectivity for adenine over mismatched bases. The analog 9-methyl-1-H-imidazo[4(5)-b] pyridine deoxyribose (dQ) is a nonpolar shape mimic of deoxyadenosine (13). Another analog,

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‡ The abbreviations used are: dQ, 9-methyl-1-H-imidazo[4(5)-b] pyridine deoxyribose; dZ, 4-methylbenzimidazole; dF, difluorotoluene deoxynucleoside; Pol γ, DNA polymerase; dFTP, difluorotoluene deoxynucleoside triphosphate; dQTP, 9-methyl-1-H-imidazo[4(5)-b]pyridine deoxyribose triphosphate; dZTP, 4-methylbenzimidazole deoxyribose triphosphate.
4-methylbenzimidazole (dZ), is also a shape mimic of adenosine like dQ but lacks a minor groove H-bond acceptor. As with dQ, dZ has been shown to be structurally similar to deoxyadenosine and to lack the ability to pair with natural bases as part of a DNA duplex (12).

To more accurately assess the role of base pair hydrogen bonding in polymerase fidelity, we have employed pre-steady state kinetic assays to determine the parameters governing incorporation of three shape mimic nucleotide analogs. In these studies we examine the human mitochondrial DNA polymerase (Pol γ), a high fidelity replicative polymerase. Our interest in Pol γ stems from its role in the toxicity of nucleoside analogs used to treat HIV infections (17, 18), and therefore, a better understanding of nucleotide discrimination by this polymerase is important in attempts to develop less toxic drugs.

In this report oligonucleotide templates containing either a deoxyadenosine shape mimic (dQ) or a deoxythymidine shape mimic (dF) were used as templates for kinetic analysis of polymerization using single turnover transient kinetic methods. Additionally, analog triphosphates difluorotoluene deoxynucleoside triphosphate (dFTP), 9-methyl-1-H-imidazo[4(5)-b]pyridine deoxyribose triphosphate (dQTP), and 4-methylbenzimidazole deoxyribose triphosphate (dZTP; dATP shape mimic) were assayed to determine the kinetic parameters of incorporation of these free nucleotides onto template nucleotides. Our results point to the significance of hydrogen bonding in DNA polymerization catalyzed by Pol γ. In the accompanying paper, we describe the essential role of hydrogen bonding in selectivity during proofreading by the 3′-5′ exonuclease (19).

**MATERIALS AND METHODS**

*Enzyme Preparation*—Expression and purification of Pol γA (catalytic subunit) and Pol γB (accessory protein) were performed as described (20, 21). Experiments to assess the polymerization using the reconstituted holoenzyme (consisting of the catalytic subunit and accessory subunit) and with the exonuclease-deficient mutant were performed as described (21–23). Protein concentrations were determined by active site titration against a known concentration of duplex DNA (21). A 1:5 molar ratio of catalytic subunit to accessory subunit was used for holoenzyme reconstitution based upon a measured $K_d$ of 35 nM (21) and a typical enzyme concentration of 50–100 nM.

*Reagents*—Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Natural nucleoside triphosphates were purchased from Sigma-Aldrich. [γ-32P]ATP was purchased from PerkinElmer Life Sciences. The nucleoside triphosphate analogs dFTP, dQTP, and dZTP were prepared as described (24, 25). The structures of dF, dQ, and dZ are presented in Fig. 2. The 45-mer oligonucleotides containing dF and dQ were also prepared as described (13, 26). All other reagents were purchased from Fisher unless otherwise noted.

*Preparation of Oligonucleotides*—The sequences of primers and templates used are listed in the Table 1. Oligonucleotides purchased from Integrated DNA Technologies were purified by preparative denaturing polyacrylamide gel electrophoresis. Lyophilized oligonucleotides were resuspended in 100 μl of deionized water and incubated at room temperature for 2 h, then 25 μl of 5× PAGE loading buffer (0.05% bromphenol blue and 0.05% xylene cyanol in formamide) was added to each oligonucleotide solution. This was then loaded in its entirety onto a 15% polyacrylamide sequencing gel. After electrophoresis, the DNA was visualized by UV fluorescence shadowing, and the bands of interest were excised. These gel pieces were then crushed and soaked in a buffer containing 1 mM EDTA and 10 mM Tris-Cl at pH 8.0 overnight at 37 °C. The crushed gel pieces were subsequently removed from the mixture by filtration through a 0.2-μm syringe filter. The volume of the solution was reduced to 0.5 ml by n-butanol extraction. The oligonu-
Discrimination values in brackets, was at a 1:1 ratio, each complex was assembled at twice the mixing of the holoenzyme-DNA complex with the nucleotide.

The holoenzyme-DNA complex was assembled to 95 °C for 5 min. Excess kinase per the manufacturer’s instructions (Invitrogen). Termination was determined by absorbance at 260 nm. Extinction coefficients at 260 and 280 nm were provided by Integrated DNA Technologies. Oligonucleotides containing analogs dF, dQ, and dZ were also purified by gel electrophoresis as previously described (13, 24, 26).

Primer strands were 5'-32P-labeled with T4 polynucleotide kinase per the manufacturer’s instructions (Invitrogen). Termination of the reaction was accomplished by heating the reaction to 95 °C for 5 min. Excess [γ-32P]ATP was removed using a BioSpin 6 column per the manufacturer’s provided instructions (Bio-Rad). Primer-template annealing was accomplished by mixing equimolar ratios of 25-mer primer and 45-mer template, heating the mixture to 95 °C, and allowing the solution to cool slowly to room temperature.

Polymerase Assay Conditions—Assays were performed at 37 °C in a reaction buffer containing 50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1 mg/ml bovine serum albumin, and 25 mM MgCl2. The holoenzyme-DNA complex was assembled in a MgCl2-free buffer and then rapidly mixed at a 1:1 ratio with the appropriate nucleoside triphosphate previously diluted into a MgCl2-containing buffer to initiate the reaction. Because mixing of the holoenzyme-DNA complex with the nucleotide was at a 1:1 ratio, each complex was assembled at twice the reported final concentration.

It should be noted that the Mg2+ concentration used in these studies (25 mM) is larger than the 2.5 mM used previously (7). This increase causes approximately a 3-fold increase in both $k_{pol}$ and the $K_a$ for nucleotide incorporation but does not change the ratio, $k_{pol}/K_a$. Accordingly, we can compare the results in this paper with those in the previous work in terms of fidelity, but individual $k_{pol}$ and $K_a$ values cannot be compared.

In this paper we have repeated the experiments to quantify the nucleotide concentration dependence of the rate of polymerization. To eliminate the complications associated with multiple enzyme turnovers, these assays were carried out under single turnover conditions, with enzymes in slight excess over DNA. Reactions were generally carried out from 0 to 100 s, although faster reactions were carried out over shorter times using an RQF-3 rapid chemical quench-flow (KinTek Corp., Austin, TX). The time courses of product formation were fit to a single exponential equation, [product] = $Ae^{-kt} + C$. Rates of polymerization determined from the single exponential fits were plotted against nucleotide concentration and fit to a hyperbola (observed rate = $k_{pol}[dNTP]/(K_a + [dNTP])$) to determine the dissociation constant, $K_a$, and the maximum rate of polymerization, $k_{pol}$, for each nucleotide combination.

Product Analysis—Products were separated from reactants on 15% denaturing polyacrylamide sequencing gels, imaged on a GE Healthcare Storm 860 PhosphorImager, and quantified using ImageQuant software (GE Healthcare).

RESULTS

In these studies we employed the three nucleoside analogs shown in Fig. 2. Single turnover kinetic assays were performed to examine the incorporation of all four natural nucleotides opposite a template dF and dQ. In addition, we examined the incorporation of dFTP opposite a template dA and dQTP and dZTP opposite a template dT. Because each of these combinations paired a normal nucleoside with a hydrophobic isostere, which likely requires desolvation of the polar partner, we also examined incorporation with two pairs of analogs, dFTP:dQ and dQTP:dF. Throughout the text we will use the shorthand, dNTP:dX, to

### TABLE 1

DNA primer and template sequences

Residues in bold represent the template base for each nucleotide incorporation.

| Residue | Primer strand sequence |
|---------|------------------------|
| 27p | 5'-GCCTGCACCGCCCTCCACACACTCACAC |
| 28p | 5'-GCCTGCACCGCCCTCCACACACTCACAC |
| 45F | 3'-GCAGGCGTCCAGGGTGGTTGATGCCAGGCAATTTAGGTAGGTCCAGGCCGTCCGAGGGTTGAGTTG |
| 45Q | 3'-GCAGGCGTCCAGGGTGGTTGATGCCAGGCAATTTAGGTAGGTCCAGGCCGTCCGAGGGTTGAGTTG |
| 45A | 3'-GCAGGCGTCCAGGGTGGTTGATGCCAGGCAATTTAGGTAGGTCCAGGCCGTCCGAGGGTTGAGTTG |
| 45T | 3'-GCAGGCGTCCAGGGTGGTTGATGCCAGGCAATTTAGGTAGGTCCAGGCCGTCCGAGGGTTGAGTTG |

* The 3’ terminal base of 28p was A, T, G, or C based upon the base pair being assayed.

### TABLE 2

Incorporation opposite nonpolar analogs

Discrimination is calculated from the ratio of $k_{pol}/K_a$ values for the correct base pair (the first in each section of the table) divided by that for each mismatch. The discrimination values in brackets, [1], are by definition.

| Template base | dNTP | $k_{pol}$ | $K_a$ | Specificity constant | Discrimination |
|---------------|------|-----------|------|---------------------|----------------|
| dF            | dATP | 110 ± 10  | 80 ± 10 | 1.4 ± 0.2           | [1]            |
| dGTP          | 0.11 ± 0.04 | 800 ± 300 | 0.00014 ± 0.00007 | 10,000        |
| TTP           | 0.072 ± 0.008 | 130 ± 20  | 0.0006 ± 0.0001    | 2500          |
| dCTP          | 0.10 ± 0.01 | 350 ± 50  | 0.00028 ± 0.00005  | 5000          |
| dQTP          | 34 ± 2   | 44 ± 5    | 0.8 ± 0.1          | 1.25          |
| dT            | 0.08 ± 0.01 | 160 ± 30  | 0.0005 ± 0.0001    | [1]           |
| dGTP          | 0.047 ± 0.009 | 170 ± 50  | 0.0003 ± 0.0001    | 1.8           |
| dATP          | 0.07 ± 0.01 | 40 ± 10   | 0.002 ± 0.0006     | 0.25          |
| dCTP          | 0.09 ± 0.02 | 290 ± 70  | 0.0003 ± 0.0001    | 1.6           |
| dFTP*         | 1.1 ± 0.02 | (250)     | (0.004)            | (0.125)       |
| dA            | dATP   | 87 ± 7    | 21 ± 0.3           | 41 ± 7        | [1]           |
| dFTP          | 9 ± 1   | 49 ± 9    | 0.18 ± 0.04        | 228           |
| dQTP          | 140 ± 10 | 3.1 ± 0.4 | 45 ± 5            | [1]           |
| dZTP          | 1.6 ± 0.1 | 460 ± 40  | 0.0035 ± 0.0004    | 13,000        |

* Incorporation of dFTP opposite dQ was assayed at 250 μM dFTP only, so the specificity constant listed is a minimum estimate.
denote the base pair formed with a given dNTP substrate reacting opposite a template containing dX.

In Figs. 3 and 4 we show representative data for a well tolerated and a poorly tolerated analog, respectively. In Fig. 3A the time course of product formation opposite a template dF (thymidine analog) is shown at concentrations of free dATP ranging from 1 to 500 μM. The data were fit to a single exponential function to obtain the rate of incorporation. In Fig. 3B, the concentration dependence of the rate was fit to a hyperbola to define $k_{pol}$ of 110 ± 10 s$^{-1}$ and a $K_d$ of 80 ± 10 μM. This surprisingly fast and efficient incorporation of dATP opposite dF is in marked contrast to the incorporation of TTP opposite the adenosine analog, dQ, shown in Fig. 4. The time course of each reaction (Fig. 4A) fit a single exponential, and the concentration dependence of the observed rate (Fig. 4B) fit a hyperbola defining $k_{pol}$ of 0.08 ± 0.01 s$^{-1}$ and $K_d$ of 160 ± 30 μM.

We also examined the fidelity of polymerization by measuring the kinetics of incorporation of mismatched bases opposite dF and dQ in the template strands. Discrimination afforded by each nonpolar analog in the template strand was then calculated as the ratio of $k_{pol}/K_d$ values obtained for the correct dNTP versus that obtained for the mismatched dNTP, defined according to the base pairing rules for the nucleotide that the analog was intended to mimic. The results from all of our assays are summarized in Table 2 and are further analyzed to evaluate the effects of analog substitutions on efficiency and specificity in Table 3 and Figs. 5 and 6.

The term $k_{pol}/K_d$ (or $k_{cat}/K_m$) serves to define both the efficiency and specificity of incorporation, but the two terms are evaluated by examining $k_{pol}/K_d$ relative to different standards. In general, efficiency is often discussed by evaluating $k_{cat}/K_m$ relative to the diffusion-limited binding rate and the physiological substrate concentration. In this report, we evaluate changes in efficiency based upon the ratio of $(k_{pol}/K_d)_\text{native}$ to $(k_{pol}/K_d)_\text{analog}$ to compare native base pairs with homologous “correct” base pairs containing one or two analogs. Changes in efficiency of incorporation relative to normal base pairs are computed in Table 3 and are shown on a free energy scale in Figs. 5 and 6.

To evaluate the effects of the analog substitutions on specificity, we compute discrimination $= (k_{pol}/K_d)_\text{correct}/
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TABLE 3
Hydrogen bonding free energy contribution to incorporation efficiency

| Base pair dNTP:template | Specificity constant \(k_{pol}/K_d\) | \(\Delta G^\prime\) kcal/mol |
|--------------------------|--------------------------------------|---------------------------|
| dATP:dT                  | 45                                   | 1                         |
| dQTP:dT                  | 0.0035                               | 5.7                       |
| dZTP:dT                  | 0.0007                               | 6.7                       |
| dATP:dF                  | 1.4                                  | 2.1                       |
| dQTP:dF                  | 0.8                                  | 2.5                       |
| TTP:dA                   | 41                                   | 3.3                       |
| dFTP:dA                  | 0.18                                 | 6.8                       |
| TTP:dQ                   | 0.0005                               | (5.4)                     |
| dFTP:dQ*                 |                                      |                           |

\((k_{pol}/K_d)_{incorrect}\) and then compare the values for discrimination obtained with analogs in the template with the values obtained using native nucleotides. Changes in specificity brought about by the nucleotide analog substitutions are illustrated on a free energy scale in Fig. 7 compared with native nucleotides.

We will use the terms discrimination, specificity, or fidelity strictly to refer to changes in \(k_{pol}/K_d\) values comparing correct and incorrect base pairs, whereas the term efficiency will be used to refer to changes in \(k_{pol}/K_d\) comparing analog correct base pairs to native correct base pairs.

Effects on Incorporation Efficiency—Fig. 5 summarizes the effect on \(k_{pol}/K_d\) on the loss of hydrogen bonding potential observed with the various analogs. The figure shows on a free energy scale the net effects on \(k_{pol}/K_d\) resolved into contributions from \(k_{pol}\) (light) and \(K_d\) (dark) relative to the values observed for the native base pair. Note that the \(k_{pol}\) free energies reflect changes in apparent transition state activation energy, whereas the numbers for \(K_d\) represent changes in ground state binding energy but in either case are computed as a \(\Delta G\) in comparing the analogs with normal nucleotides.

Fig. 5A compares the results obtained with each analog base pair to those seen with a normal dATP:dT base pair. In this figure the magnitude of the bar defines the apparent free energy loss with each analog. For example, it can be seen that for dQTP:dT there is a loss of ~3 kcal/mol in ground state binding free energy and 2.7 kcal/mol in apparent free energy contributions to the maximum rate, \(k_{pol}\), giving a net loss of 5.7 kcal/mol in \(k_{pol}/K_d\). In contrast, dATP:dF shows about a 2 kcal/mol loss in ground state binding free energy but very little effect on \(k_{pol}\). In general for this series, the loss of hydrogen bonding potential leads to 2–3 kcal/mol reduction in ground state binding free energy, whereas effects on the maximum rate of polymerization are quite variable, from negligible (dATP:dF) to nearly 4 kcal/mol (dZTP:dT).

In Fig. 5B, we show \(\Delta G\) values for changes in efficiency for base pair mimics of TTP:dA. These data show marked reductions in both ground state binding (3 kcal/mol) and maximum rate of incorporation (2–4 kcal/mol) due to the substitution of dQ for dA in the template. In addition, the vertical alignment of columns in Fig. 5B is correlated with Fig. 5A to allow comparison with the analog in the incoming dNTP rather than the template; for example, we compare TTP:dQ with dQTP:dT in the first column. The trends across this series are consistent between Figs. 5, A and B, indicating the efficiencies of incorporation follow the trend (TTP:dQ < dQTP:dT) < (dFTP:dQ) < (dFTP:dF) < (dFTP:dA) < (dATP:dF).

Effects on Fidelity—The effects of the analog substitutions on fidelity of Pol γ are summarized in Fig. 7. The net effect on discrimination is shown on a free energy scale, and the contributions to the apparent ground state binding (\(K_d\)) and maximum rate of polymerization (\(k_{pol}\)) are resolved as the dark and light sections of each bar, respectively. In this figure a larger bar represents better discrimination. The two figures on the left

FIGURE 5. Thermodynamic effects of analog substitutions on efficiency. Contributions to the enzyme efficiency, proportional to \((k_{pol}/K_d)\), are shown on a free energy scale (kcal/mol, 37 °C). We show the effects on \((k_{pol})\) (light) and \(K_d\) (dark) contributing to the net loss in efficiency. The free energy differences were computed as \(\Delta G = -RT\ln(K_{pol,native}/K_{pol,analog})\) and \(\Delta G^\prime = -RT\ln(K_{pol,native}/K_{pol,analog})\). The sum of the light and dark bars defines the net free energy difference leading to changes in \(k_{pol}/K_d\) in comparing native base pairs with those involving one or more analogs. A, the constants for the analog-containing base pairs are compared with the native dATP:dT base pair. B, constants for the analogs are compared with the native TTP:dA base pair. In each figure a larger value implies a greater cost as a result of analog substitutions. Note that the values for dFTP:dQ are only an estimate based upon a single concentration.
DISCUSSION

The most striking results from these of experiments are the relatively efficient incorporation dATP and dQTP opposite dF, and moderate, although significantly reduced, discrimination opposite dF in the template. This is in marked contrast to the extremely slow, inefficient incorporation and the total absence of discrimination among various dNTPs, when dQ is present in the template. These two extremes may define the upper and lower limits for the role of hydrogen bonding in DNA polymerase efficiency and selectivity. In one case (dQ), hydrogen bonds are absolutely essential for efficient and specific DNA replication in that all dNTPs bind weakly and are incorporated slowly.
opposite dQ as the templating base. On the other hand, dF sets a lower limit on the magnitude of the role of hydrogen bonds, suggesting that hydrogen bonding contributes approximately a third of the free energy toward efficiency and specificity of nucleotide incorporation.

Alternatively, the lower efficiency and selectivity seen with dQ could be due to steric effects. It was previously observed that dQ is a poor substrate for other A-family polymerases, just as it is here (27), and it has been argued that the steric bulk of the extra hydrogen of dQ renders it substantially larger than dA. At the N1 analogous position, the C-H group of dQ adds a chemical bond (length 1.1 Å) directly in the center of the base pair face of the analog (see Fig. 2). Recent studies have shown that increases of as little as 0.5 Å in steric size at the base pair face of a base can lead to 2–3 orders of a magnitude drop in incorporation efficiency with other A family polymerases (9, 28). Therefore, the reduced efficiency and specificity observed for incorporations opposite dQ may be due to the steric repulsion due to the C-H in place of N1 in adenine, and therefore, studies with dQ may overestimate the contributions attributable to hydrogen bonding.

In contrast, structural and kinetic data suggest that dF is a reasonable thymine analog, and the small changes in observed efficiency and selectivity define a minimal role of hydrogen bond formation during polymerization. As described below, this “minimal” role is still significant, arguing that the hydrogen bonds in the dA:dT base pair contribute ~2.4 kcal/mol toward polymerase efficiency and specificity. Moreover, these effects are manifested largely in the ground state binding, not the rate of polymerization. The data obtained with dF as a templating base support our previous assessment suggesting that hydrogen bonding effects are manifested largely in the ground state binding, whereas the maximum rate of polymerization may be dependent upon the shape of the base pair (2). However, this conclusion is valid only in the case of dF as a templating base, and the generality breaks down when dFTP is the incoming nucleotide.

In general, the analog in the template strand leads to a smaller loss of activity than it does as the incoming dNTP. This observation may imply that hydrogen bonds and the cost of desolvation are more important in binding and aligning the incoming dNTP at the catalytic reaction center than in the templating base.

In the formation of each mixed base pair involving one native base and one nonpolar isostere, one expects an energy cost due burying a hydrogen bond donor or acceptor against the hydrophobic surface of the analog. Fig. 6 illustrates the thermodynamic consequences of pairwise analog substitutions for a series of analog pairs mimicking dATP:dT. ΔΔG values were computed from $k_{pol}/K_d$ values relative to the dQTP:dF nonpolar isostere, thereby showing the consequence of sequential addition of hydrogen bonding groups to one or both bases. Starting with dQTP:dF, substitution of dQTP with dATP to form dATP:dF increases efficiency slightly (0.4 kcal/mol). However, alternative substitution of dF with dT to form dQTP:dT entails a significant energy cost (3.3 kcal/mol), which is overcome in returning to full hydrogen bonding potential (dATP:dT). Similarly, comparing dATP:dF with dATP:dT shows the effect of restoring hydrogen bonds to the base pair (2.1 kcal/mol). This figure shows that the thermodynamic consequence of burying hydrogen bonding groups is asymmetric. This asymmetry is also seen when comparing dQTP:dF with dFTP:dQ (Table 2).

It has been previously shown that dFTP is structurally very similar to TTP (25, 26). The difference in dFTP:dA pairing efficiency (relative to the reverse, dATP:dF) was also seen previously with the Klenow polymerase (25) and is suggestive of asymmetry in the two strands of the incipient base pair; for example, there may be differences in solvation in the template base and the base of the incoming dNTP, leading to differences in the cost of desolvation upon pairing. The cost of desolvation of adenine in the dATP:dF pairings may be relatively small, consistent with calculations showing adenine to be the most weakly solvated of the DNA bases, whereas desolvation of thymine (as in dQTP:dT) is expected to be energetically more costly (29).

The analogs dQTP and dZTP are structurally very similar and have been previously shown to be structurally similar to dATP. It was believed that this structural similarity would allow these two molecules to be employed efficiently and are the best available non-H-bonding shape analogs of dATP. The kinetic data argue, however, that dQTP and dZTP are not efficient mimics of the structure of dATP with Pol γ. This could well be due to the higher cost of desolvation of the partner dT and possibly to the added bulk of the proton due to substitution at N1 to form dZ and dQ. However, mutations designed to overcome steric hindrance against dZ by Klenow failed to restore catalytic efficiency (27).

The 5-fold difference in the specificity constant for incorporation of dQTP and dZTP is interesting because the only difference in the structures of these two molecules is the presence of a nitrogen atom at the 1 position of the purine structure in dQTP. It has been previously posited that there are interactions between the polymerase and the minor groove of the DNA duplex (27). The presence of this nitrogen atom at position 1 of the purine ring has a beneficial effect on the incorporation kinetics, and perhaps this is due to favorable interactions between the enzyme and dQTP due to this nitrogen. It is possible that hydrogen-bonded interactions with the enzyme and the minor groove of the DNA duplex may be the cause of this difference in incorporation between these two very similar molecules (13, 30, 31). Analysis of mutations with dQ and dZ analogs revealed an arginine (Arg-668 in Klenow) that may participate in a hydrogen bond with the minor groove of the primer-terminal base (27).

The efficiency of incorporation of dQTP:dF relative to dATP:dT is reduced by only 2.5 kcal/mol, with 1.6 kcal/mol due to changes in $K_f$ and 0.9 kcal/mol due to changes in $k_{pol}$. It has been hypothesized that the rate of chemistry reflects the geometric shape of the base pair formed at the active site and that the $K_f$ for nucleotide binding reflects the contribution due to hydrogen bonding (2). Applied to this simple model, these data would suggest that there is a 1.6 kcal/mol loss of ground state binding energy due to the loss of hydrogen bonding and a 0.9-kcal/mol loss due to changes in the geometric shape of the base pair observed with the analogs. However, the high efficiency observed with the dQTP:dF
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There are several factors that can affect selectivity of nucleotide incorporation by a DNA polymerase, including base pair hydrogen bonding, base pair size and shape, and base stacking interactions. In addition, interactions at the DNA polymerase active site can alter each of these factors. In this study we have characterized the effect of three nonpolar nucleotide analog substitutions in an effort to determine the magnitude of the contribution to fidelity afforded by base pair hydrogen bonding. We have shown that the human mitochondrial polymerase catalyzes efficient incorporation with a significant level of discrimination against mismatches opposite dF in the template, although the magnitude of discrimination is 46-fold lower than seen with the corresponding natural nucleotide (dT). We have also shown that Pol γ catalyzes very inefficient and inaccurate incorporation opposite a template dQ, although this may be due to steric effects. Nonetheless our results demonstrate that base pair hydrogen bonding is a substantial contributor to the fidelity of Pol γ.

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