Importance of Terminal Base Pair Hydrogen-Bonding in 3′-End Proofreading by the Klenow Fragment of DNA Polymerase I†

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ABSTRACT: We describe studies aimed at evaluating the physical factors governing the rate of 3′-end proofreading by the Klenow fragment of *E. coli* DNA polymerase I. Two nonpolar deoxynucleoside isosteres containing 2,4-difluorotoluene (F) and 4-methylbenzimidazole (Z), which are non-hydrogen-bonding shape mimics of thymine and adenine, respectively, are used to investigate the effects of base pair geometry and stability on the rate of this exonuclease activity. Steady-state kinetics measurements show that complementary T·A base pairs at the end of a primer−template duplex are edited 14−40-fold more slowly than mismatches. By contrast, a 3′-end T residue in a T·Z pair is edited at a rate equivalent to that of natural base mismatches despite the fact that it resembles a T·A pair in structure. Similarly, the A in an A·F pair is edited as rapidly as a mismatched pair despite its close structural mimicry of an A·T pair. Interestingly, when the base pairs are reversed and F or Z is located at the 3′-end, they are edited more slowly, possibly implicating specific interactions between the exonuclease domain and the base of the nucleotide being edited. Finally, thermal denaturation studies are carried out to investigate the relationship between editing and the ease of unwinding of the duplex. The rapid editing of bases opposite F or Z residues at the duplex terminus seems to correlate well with the stability of these base pairs when placed in a context resembling a primer−template duplex. In general, the rate of 3′-end editing appears to be governed by the rate of fraying of the DNA terminal pair, and base pair geometry appears to have little effect.

The high fidelity of information transfer that occurs during replication is influenced by a number of factors. Among these are the fidelity of nucleotide insertion, the fidelity of base pair extension, the fidelity of DNA proofreading, and the correction of errors by repair mechanisms (*1*−*4*). The first three of these steps are governed by polymerase enzymes, which possess activities not only for synthesis of DNA but also (commonly) for 3′-end editing. The 3′ exonuclease (exo) domain of the Klenow fragment of *E. coli* DNA polymerase I (Kf)[1] is found in the amino terminal region of the same polypeptide chain as the polymerase domain (*5*). Kinetic analysis of Kf shows that it edits 1 base pair for every 10−15 that are synthesized (*6*). The rate-limiting step in editing during DNA polymerization for T4 DNA polymerase is the unwinding and translocation from polymerase to the exo site as determined using the 2-aminopurine fluorescence probe (*7*). It is not yet clear whether Kf has a similar kinetic barrier. X-ray crystal structures of the Kf enzyme (*8*, *9*) and related polymerases (*10*) show that the exo domain is located approximately 30 Å from the polymerase cleft. For editing to occur, at least four base pairs of the duplex in the polymerase cleft must unwind and the primer strand must then be translocated to the exo domain (*11*). A crystal structure of an editing complex of Kf DNA polymerase with an 11-base-pair duplex DNA substrate containing a 3′ overhanging single strand showed that the 3′-terminus was bound to the 3′-5′ exonuclease active site (9). The structural data revealed that the protein makes hydrophobic contacts with the unstacked nucleotide bases and hydrogen bonds with the sugar−phosphate backbone.

Recent studies have focused on the kinetics of the 3′ exonuclease activity of the Kf polymerase to determine what factors govern the enzyme's decision to edit rather than extend a given base pair, and its preference to excise mismatched base pairs more rapidly than correctly paired termini. The exonuclease binding site functions solely as a single-stranded exonuclease, and therefore makes no direct selection for a mispaired base. Two possible mechanisms for selectivity in editing have been proposed. The first model proposes that recognition of a mismatched base pair geometry at the polymerase binding site could cause the translocation of the DNA terminus to the exonuclease binding site (*12*, *13*). A second model suggests that there may be increased melting capacity with mismatches at the 3′-terminus, causing the DNA to be edited more easily because it exists more often in a single-strand configuration (*12*−*15*). Different lines

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[1] Abbreviations: F, 2,4-difluorotoluene deoxynucleoside; Z, 4-methylbenzimidazole deoxynucleoside; Kf, Klenow fragment of *E. coli* DNA polymerase I; 2AP, 2-aminopurine; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; T_m, melting temperature; 2D-NMR, two-dimensional nuclear magnetic resonance.

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of experimental data support the melting model. Proofreading experiments carried out with T4 DNA polymerase (*14*−*16*), DNA polymerase III (*12*), and Kf (*17*) show a marked increase in exonuclease activity with duplex DNA as temperature is increased. Evidence from pre-steady-state kinetics measurements studying excision of 2-aminopurine (2AP) by T4 DNA polymerase also lends support to this model (*18*). A "stable" 2AP·T base pair is hydrolyzed much more slowly than an unstable 2AP·C mismatch; however, a terminal 2AP·T placed in an A·T-rich DNA environment is hydrolyzed more rapidly than a 2AP·C pair in a C·G-rich DNA environment, showing the importance of DNA stability for editing efficiency.

A third, mixed, mechanism for 3′ editing has been proposed wherein the selectivity for editing is attributed partially to the melting capacity of the DNA and partially to the polymerase binding site, promoting movement of the DNA into the exonuclease site by rejecting aberrant primer termini. Time-resolved fluorescence anisotropy techniques have been used to determine the fractional occupancies of the polymerase and 3′−5′ exonuclease sites of Kf by the DNA (*19*). The free energy difference for partitioning of correct base pairs versus mismatches is found to be −0.6 to −0.7 kcal/mol, whereas the difference in melting free energies between correct base pairs and mismatches is calculated to be −0.2 kcal/mol (*20*). The difference between the melting and partitioning free energies suggests that both weaker binding at the polymerase binding site and greater melting capacity contribute to the selectivity in the proofreading process.

One limitation of studies involving natural bases is that it is difficult to distinguish between geometric arguments and base pair stability effects, because a natural mismatch both is destabilizing and has noncanonical geometry. To address such issues more directly, we have therefore undertaken a study of polymerase activities using nucleoside analogues, nonpolar nucleoside isosteres, which mimic size and shape as closely as possible, but which lack hydrogen-bonding ability (*21*, *22*). Experiments measuring nucleotide insertion by the Kf enzyme have shown that these analogues can be efficiently and selectively incorporated into DNA, despite their lack of Watson−Crick hydrogen-bonding groups. Those studies have provided evidence that base pair geometry, enforced by the tightly constrained polymerase active site, may play as large a role as base−base hydrogen bonds in determining the selectivity of nucleotide insertion (*23*−*25*).

It is this same polymerase cleft in which the partitioning between polymerase and exonuclease sites occurs. As a result, non-hydrogen-bonding nucleoside isosteres might allow one to distinguish between geometric and base-pairing effects more directly than was previously possible. When paired with specific natural bases, these compounds are able to mimic closely the natural base pair geometry (*26*). However, since they lack hydrogen-bonding ability, they form unstable pairs with their natural partners. Here we report that the pairing partners of such analogues are edited rapidly despite this structural mimicry. The results suggest that the melting capacity of the DNA terminus is the chief factor determining the rate at which it is edited by the Klenow polymerase and, more specifically, suggest that the fraying of the terminal base pair (limited by hydrogen bonds) plays an important role.

MATERIALS AND METHODS

Oligodeoxynucleotide Synthesis. The phosphoramidite derivatives of F and Z were prepared as described (*22*, *27*). DNA oligonucleotides were synthesized on an Applied Biosystems 392 synthesizer using standard β-cyanoethylphosphoramidite chemistry. Oligomers were purified by preparative 20% denaturing polyacrylamide gel electrophoresis and were quantitated by absorbance at 260 nm. Molar extinction coefficients were calculated by the nearest-neighbor method. Values for oligonucleotides containing the nonnatural residues F and Z were derived from the sum of the molar extinction coefficient for F or Z (measured to be 1200 and 5620 at 260 nm, respectively) and the calculated values of the adjacent sequences.

Qualitative Proofreading Reactions. Primer 5′-termini were labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. The labeled primer was annealed to the template in a buffer of 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 50 mg/mL BSA. Proofreading reactions were started by mixing equal volumes of a solution containing the DNA and a solution containing *E. coli* DNA polymerase Klenow fragment (Kf) (Amersham-USB). The solution containing Kf was prepared by dilution from the stock solution in the same annealing buffer as the DNA. The reaction mixture was incubated at 25 °C, and at appropriate time points, aliquots were removed and quenched by adding 1.5 volumes of stop buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and bromophenol blue). The final concentrations were 25 and 50 nM, respectively, for primer/template and 300 nM for Kf. The reactions were analyzed by running quenched reaction samples on 15% denaturing polyacrylamide gels.

Steady-State Kinetics. Steady-state kinetics for proofreading experiments were carried out as described (*12*, *28*). The reaction conditions used were the same as for the qualitative editing studies. Primer and template DNA strands at concentrations ranging from 5 to 50 nM (except for the base pairs T·A and F·A (5−150 nM)) were annealed using the same buffer as in the qualitative studies. The concentration of labeled DNA primer was taken into account, and it was assumed that all the DNA primer from the labeling reaction was recovered after ethanol precipitation. Reactions were initiated by adding the Kf to a final concentration of 0.002 u/μL (3 nM). The reaction mixtures were incubated at 25 °C for 3 min for all base pairs, except for Z·Y pairs (6 min) and T·A and F·A pairs (60 min), and were terminated by adding 1.5 volumes of stop buffer. Extents of reaction were determined by running quenched reaction samples on a 15% denaturing polyacrylamide gel. Relative velocities were calculated on the basis of eq 1, where [S] is the concentration

$$V = \frac{[S]I_1/(I_0 + I_1)}{t} \quad (1)$$

of substrate (primer−template), t is the reaction time, and I_0 and I_1 are band intensities of the original primer (14mer) and the excised product (13mer), respectively.

Thermal Denaturation Studies. Solutions for the thermal denaturation studies contained a self-complementary oligomer in a buffer solution of 1 M NaCl, 0.1 mM EDTA, and 10 mM Na·phosphate (pH 7.0). After the solutions were

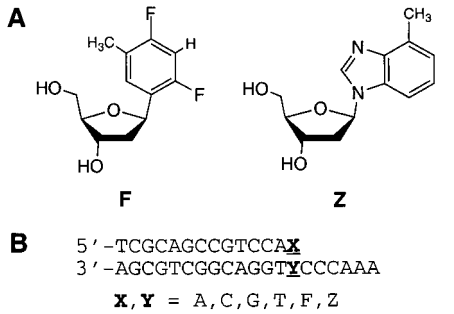

FIGURE 1: Structures and sequences in this study: (A) structures of nonpolar deoxynucleoside analogues 2,4-difluorotoluene (F) and 4-methylbenzimidazole (Z); (B) sequences of primer−template duplexes used in the proofreading experiments.

prepared, they were heated at 90 °C for 5 min and allowed to cool slowly to room temperature prior to the melting experiments. The sequences were monitored at 280 nm. Thermodynamic parameters were determined by plotting $1/T_m$ vs ln [oligonucleotide] using at least five different concentrations for each duplex. All of the measured complexes generated van't Hoff plots with good linear fits and displayed apparently two-state melting behavior.

RESULTS

Exonuclease Activity of Kf on Base Pairs Containing F and Z Nonpolar Isosteres. Two nonpolar nucleoside isosteres of thymidine and deoxyadenosine, difluorotoluene deoxynucleoside (F) and 4-methylbenzimidazole deoxynucleoside (Z) (Figure 1a), respectively, were used in these studies. To determine qualitatively the efficiency of editing of base pairs containing F and Z, we selected DNA sequences where these nonpolar bases are placed at the 3′-termini, either in the primer or in the template strand (Figure 1b). The sequence context used was previously shown to give good kinetic behavior with the Kf enzyme (*29*). The reactions were carried out with an excess of Kf over DNA substrate to assume full binding of DNA to the polymerase for all DNA sequences. The products were analyzed by denaturing polyacrylamide gel electrophoresis.

We examined qualitatively the proofreading activity of Kf when either F or Z was placed in the template at the 3′-terminus. All the pairs with natural bases were edited almost completely in less than 30 s, giving the 13mer band that corresponds to an A·T base pair, which is subsequently excised much more slowly (Figures 2 and 3). All the X·F and X·Z base pairs (X is the base in the primer and F or Z is the base in the template) behave similarly to the control mismatches C·A and C·T, whereas the normal base pairs A·T and T·A were edited more slowly, and were not completely excised even after 4.5 min. Both the A·F and T·Z base pairs, which are isosteric with A·T and T·A, were cleaved as if they were mismatches, even though they apparently mimic the Watson−Crick geometry. The nature of the base paired with F or Z made no difference in the editing rate.

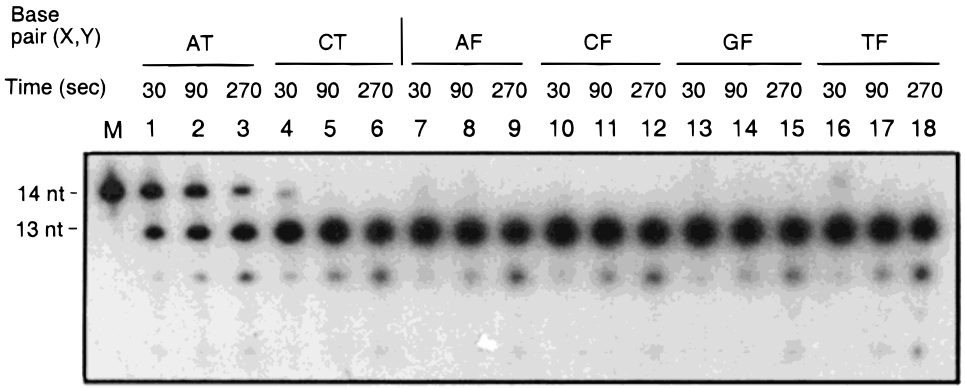

FIGURE 2: Autoradiogram of denaturing PAGE gel showing the editing experiment on base pairs with F in the template strand (X·F). The data were taken at 25 °C using KF 0.2 u/mL, and 25/50 nM primer−template DNA, and the reactions were stopped after 0.5, 1.5, and 3 min.

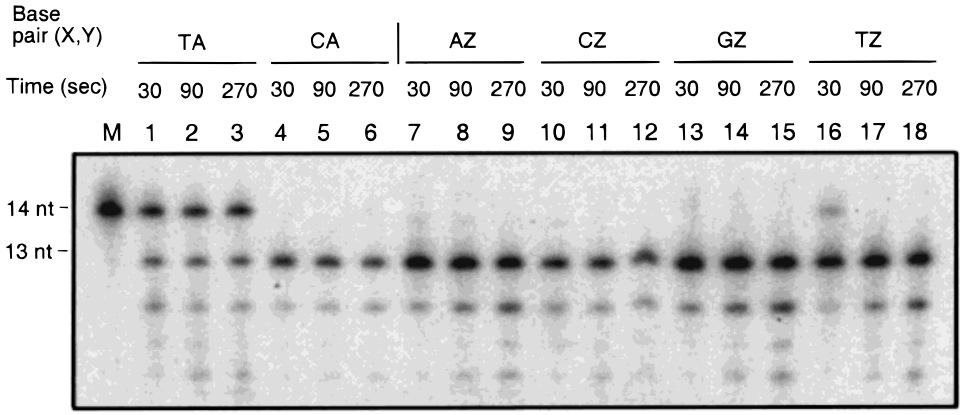

FIGURE 3: Autoradiogram of denaturing PAGE gel showing the editing experiment on base pairs with Z in the template strand (X·Z). The conditions used were the same as in Figure 2.

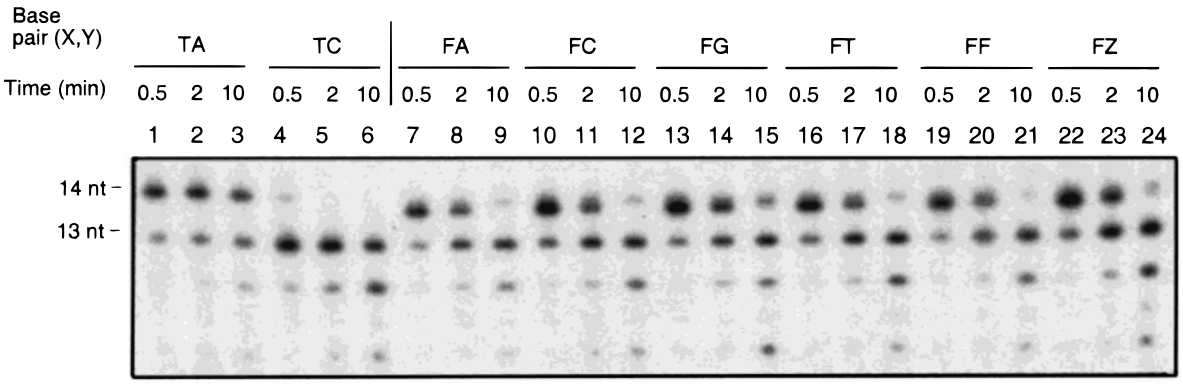

FIGURE 4: Autoradiogram of denaturing PAGE gel showing the editing experiment on base pairs with F in the primer strand (F·Y). The conditions used were the same as in Figure 2.

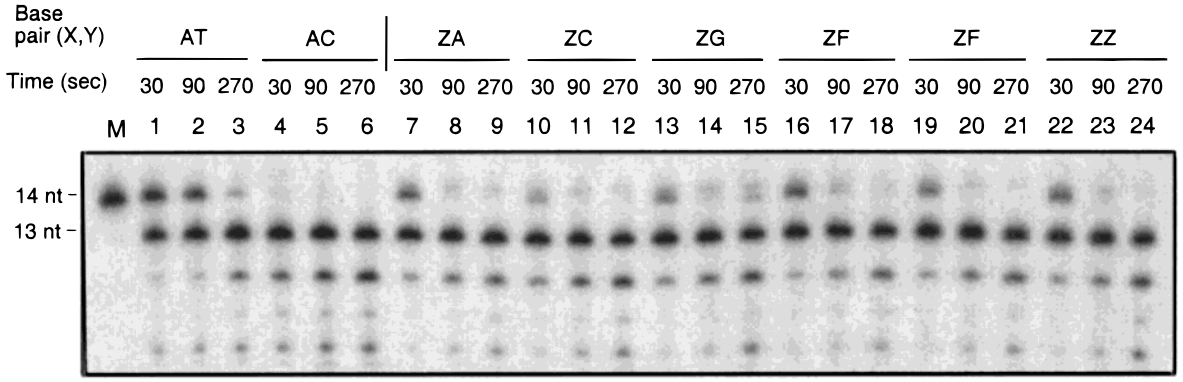

FIGURE 5: Autoradiogram of denaturing PAGE gel showing the editing experiment on base pairs with Z in the primer strand (Z·Y). The conditions used were the same as in Figure 2.

When F was placed in the primer at the 3′-terminus, the editing of all the F·Y pairs proceeded much more slowly than for the control mismatch T·C and was not complete after 10 min (Figure 4). The control base pair T·A was cleaved even more slowly than the F·Y pairs, and more than 50% of the 14mer was left after 10 min. Importantly, no selectivity between F·A and all the other F·Y pairs was observed, regardless of the pyrimidine·purine or pyrimidine·pyrimidine structural form.

In the case of Z situated in the primer at the 3′-terminus, the Z in all Z·Y pairs was excised more rapidly than in the case of F, and only 5−10% remained after 30 s (Figure 5). The control mismatch A·C was cleaved even faster than Z·Y pairs, whereas the A·T pair was edited more slowly, and after 4.5 min a small percentage remained. Again, there was no appreciable difference in efficiency of editing between Z·T and any of the other Z·Y base pairs. Thus, for both the F·Y and Z·Y base pairs, the editing seems to be slower than for a mismatch and faster than for a normal pair. It is important to note than in these two cases a nonnatural nucleotide is being removed in the exonuclease active site, and the rate may be affected not only by the geometry or melting capacity of the pair but also by the direct contacts between the enzyme and the nonnatural substrate.

Steady-State Kinetic Parameters for Exonuclease Activity on Base Pairs Containing F and Z Nonpolar Isosteres. To quantitate the differences observed in editing of base pairs containing analogues F and Z, and to compare them with mismatched and correct base pairs, we carried out steady-state kinetic analysis. Conditions were determined wherein the reaction velocity was constant over the period of time measured. Reaction velocities were measured at various substrate concentrations (see the Materials and Methods), and the values for V_{max} and K_m were obtained by plotting [S]/V vs [S] (the Hanes−Woolf form). The kinetic parameters obtained for base pairs containing F or Z and for natural base pairs are summarized in Table 1.

Table 1: Kinetic Parameters for the Proofreading Activity of a Primer Terminus Nucleotide by KF polymerase I

primer (X)	template (Y)	V_{max} (nM/min)	K_m (nM)	V_{max}/K_m	rel eff
C	T	3.2 (±0.5)	12.3 (±1.0)	0.26 (±0.04)	0.7
G	T	1.6 (±0.3)	5.5 (±0.8)	0.29 (±0.07)	0.7
T	T	2.4 (±0.3)	17.6 (±1.2)	0.14 (±0.02)	0.4
A	A	5.5 (±1.6)	15.1 (±8.7)	0.4 (±0.2)	1
C	A	4.5 (±1.2)	17.3 (±5.2)	0.3 (±0.1)	0.8
G	A	1.7 (±0.3)	5.7 (±1.9)	0.3 (±0.1)	0.8
T	A	1.83 (±0.2)	170 (±34)	0.01 (±0.002)	0.03
A	F	4.8 (±0.6)	13.4 (±2.7)	0.36 (±0.08)	0.9
C	F	4.5 (±1.2)	18.6 (±4.6)	0.24 (±0.09)	0.6
G	F	3.4 (±0.7)	13.5 (±5.4)	0.3 (±0.1)	0.8
T	F	1.6 (±0.3)	4.9 (±1.0)	0.33 (±0.09)	0.8
A	Z	6.7 (±2.9)	17.7 (±9.0)	0.4 (±0.3)	1
C	Z	3.9 (±0.4)	14.1 (±3.3)	0.28 (±0.07)	0.7
G	Z	3.4 (±0.2)	10.8 (±2.7)	0.32 (±0.08)	0.8
T	Z	2.1 (±0.3)	7.7 (±2.5)	0.27 (±0.09)	0.7
F	A	2.3 (±0.2)	190 (±60)	0.01 (±0.003)	0.03
Z	A	4.4 (±0.3)	45.0 (±0.2)	0.10 (±0.007)	0.3
Z	T	3.5 (±0.5)	28.3 (±5.9)	0.13 (±0.03)	0.3
Z	F	6.6 (±1.5)	51.3 (±12)	0.13 (±0.04)	0.3
Z	Z	3.1 (±0.7)	22.0 (±4.3)	0.14 (±0.04)	0.4

Our kinetic data for natural DNA mismatches are in good agreement with published values in a similar sequence (*28*). The relative efficiency (V_{max}/K_m) of proofreading excision

for all the natural mismatches exhibits only small variations up to 2.9-fold, with T·T being the slowest mismatch to be cleaved. In the case of a correctly matched T·A base pair the relative efficiency is 14−40-fold lower than in the mismatch cases, which agrees with the known preference toward mismatches previously reported (*17*, *29*). For base pairs containing F or Z in the template strand, the V_{max}/K_m is very similar to that obtained for all the mismatches (except T·T). The editing of A·F and T·Z, which can adopt a geometry close to that of a standard Watson−Crick base pair (*26*), is as efficient as all the other base pairs X·F and X·Z.

When F is in the primer strand, the relative editing efficiency is 14−40-fold lower than that of the natural mismatches, which suggests that some important noncovalent interactions are missing between F and the exonuclease active site. In the related case of Z in the primer, however, the V_{max}/K_m is only 1.7−4-fold lower than those of the mismatches, and very similar to that of the T·T base pair, suggesting that whatever interactions F might be missing in the exo site, Z does not suffer as strongly (see below). Notably, within the Z·X series there is no observed difference in editing due to the different geometries these pairs display. It is worth noting that the steady-state kinetic results allow us to assess only the slowest step of the reaction, and it is possible that the nonpolar analogues cause the rate-limiting step to change relative to what occurs with natural bases, especially when F and Z are edited.

Thermodynamic Stability of Base Pairs at the Primer−Template Terminus. We next examined the pairing properties of nucleosides F and Z when placed in blunt-ended self-complementary duplexes, to try to correlate the melting capacity of the different DNAs with their editing rates. We also examined DNA sequences with two additional dangling cytosines at the 5′-end to more closely mimic the structure of a primer−template complex. We examined base pairs involving analogues F and Z as well as several natural mismatches and correct base pairs. Thermodynamic stabilities of the duplexes were measured by UV-monitored thermal denaturation studies at pH 7.0 in the presence of 1 M Na^+, 10 mM NaH_2PO_4, and 0.1 mM EDTA (Tables 2 and 3).

In the case of the blunt-ended natural strands (Table 2), the purine·pyrimidine mismatches are less stable than A·T or T·A base pairs, with T_m values 4−6 °C lower and free energies (37 °C) 0.3−0.45 kcal·mol^{-1} less favorable, whereas a purine·purine mismatch, G·A, is as stable as T·A, and 0.15 kcal·mol^{-1} less stable than A·T. These differences between mismatches and correct pairs are lower than those seen for similar mismatches when placed in the middle of the strand, where pairing selectivity is 4−5 kcal·mol^{-1} (*27*). All the base pairs containing F and Z at the termini in blunt-ended sequences were more stable than A·T and T·A base pairs, with T_m values 2−5 °C higher and free energies (37 °C) 0.1−0.4 kcal·mol^{-1} more favorable. The single exception is the G·Z case, which has stability equal to that of the A·T case.

The oligonucleotides with 5′ dangling residues that contain natural pairs at the termini show an increase in stability as compared to the blunt-end sequences (Table 3), consistent with previous studies of the effects of dangling nucleotides (*30*, *31*). By contrast, A·F and T·Z are less stable in the duplexes with dangling residues than in the blunt-end sequences, with T_m values 2 °C lower and free energies (37 °C) 0.2−0.3 kcal·mol^{-1} less favorable. This indicates that base pairs of F and Z with natural polar bases are less stable when shielded from the solvent in the middle of the DNA strand, an effect observed previously (*22*, *27*).

Table 2: Free Energies and Melting Temperatures for Duplexes Containing Nonpolar Base Pairs F and Z at the Terminus of Blunt-End Sequences

DNA duplex	T_m [a]	$-\Delta G°_{37}$ [b]	$-\Delta G°_{37}$ [c]	$\Delta\Delta G$[d]
5′-**T**CGCGCG**A** 3′-**A**GCGCGC**T**	54.35	10.3	10.3	--
5′-**A**CGCGCG**T** 3′-**T**GCGCGC**A**	54.96	10.0	10.0	-0.15
5′-**C**CGCGCG**A** 3′-**A**GCGCGC**C**	50.24	9.5	9.4	-0.45
5′-**C**CGCGCG**T** 3′-**T**GCGCGC**C**	49.21	9.1	9.2	-0.55
5′-**A**CGCGCG**G** 3′-**G**GCGCGC**A**	54.11	10.1	10.0	-0.15
5′-**F**CGCGCG**A** 3′-**A**GCGCGC**F**	58.22	10.6	10.8	0.25
5′-**Z**CGCGCG**T** 3′-**T**GCGCGC**Z**	56.51	10.5	10.6	0.15
5′-**Z**CGCGCG**G** 3′-**G**GCGCGC**Z**	53.77	10.0	10.1	-0.1
5′-**F**CGCGCG**Z** 3′-**Z**GCGCGC**F**	59.09	10.9	10.7	0.2
5′-**Z**CGCGCG**F** 3′-**F**GCGCGC**Z**	57.06	10.4	10.4	0.05

[a] Concentration of DNA strand 7.5 μM. [b] Free energies determined by averaged values from each fitting curve (kcal·mol^{-1}). [c] Free energy determined from a van't Hoff plot (ln C vs $1/T_m$) (kcal·mol^{-1}). [d] $\Delta\Delta G = (\Delta G - \Delta G_{AT})/2$.

Table 3: Free Energies and Melting Temperatures for Duplexes Containing Nonpolar Base Pairs F and Z in Sequences with Two Dangling Cytosines

DNA duplex	T_m [a]	$-\Delta G°_{37}$ [b]	$-\Delta G°_{37}$ [c]	$\Delta\Delta G$[d]
5′-CC**T**CGCGCG**A** 3′**A**GCGCGC**T**CC	58.29	11.4	11.4	--
5′-CC**C**CGCGCG**A** 3′**A**GCGCGC**C**CC	50.94	9.5	9.6	-0.9
5′-CC**F**CGCGCG**A** 3′-**A**GCGCGC**F**CC	55.93	10.3	9.9	-0.75
5′-CC**Z**CGCGCG**T** 3′-**T**GCGCGC**Z**CC	54.26	10.0	10.2	-0.6

[a] Concentration of DNA strand 7.5 μM. [b] Free energies determined by averaged values from each fitting curve (kcal·mol^{-1}). [c] Free energy determined from a van't Hoff plot (ln C vs $1/T_m$) (kcal·mol^{-1}). [d] $\Delta\Delta G = (\Delta G - \Delta G_{AT})/2$.

DISCUSSION

The present data show that, of the six mismatches studied, all are edited within a narrow range of rates; the fastest (A·A) is processed only 2.9-fold more efficiently than the slowest (T·T). Similar efficiency is observed for the cases with F and Z in the template, with an even smaller range of rates (up to 1.7-fold). Interestingly, all the "mismatches" can be divided into groups with similar V_{max}/K_m ratios, where each group possesses the same base being excised. Thus, the general order of editing is A·Y > G·Y ≈ T·Y > C·Y, with an average V_{max}/K_m of 0.39 for the fastest A·Y and an average V_{max}/K_m of 0.27 for the slowest C·Y, with T·T as the only exception, being cleaved more slowly than its related T·Y pairs. This small effect could be due to the actual

preference of the exonuclease site for one substrate versus the others; for example, purines may interact with the exo site residues more favorably because of their increased stacking ability, or by their common N7 groups. This might also explain why T is edited more rapidly than C, since thymine O4 is placed similarly to purine N7.

Our exonuclease data show that isosteric pairs A·F and A·T are edited at very different rates. Similarly, the T·Z pair is edited 27-fold more rapidly than T·A despite the possibility that it is also isosteric to the hydrogen-bonded natural pair. This confirms that rapid proofreading, as observed for mismatches, can occur in the absence of DNA structural distortion. Indeed, since we see relatively little variation between editing of A·F, which likely closely resembles a canonical pair, and editing of G·F, C·F, and T·F, which cannot, we conclude that geometry appears to play little or no role in the rate of proofreading. It should be noted that although there are no data on the structure of the A·F pair in this specific context, we have examined this pair in the context of the center of a DNA duplex by 2D-NMR methods, and it was found to be virtually indistinguishable in structure from an A·T base pair in the same context (*26*).

When the thermodynamic data obtained in the blunt-ended sequences are compared with the relative efficiency of proofreading measured by steady-state kinetics, the stability differences between the mismatches and the normal base pairs are too small to explain the relative editing efficiency. For example, the mismatch G·A is as stable as a T·A base pair, whereas the efficiency of editing G·A is 30-fold higher than that for T·A. Similarly, the base pairs containing F and Z are more stable than the A·T and T·A base pairs (because of their superior stacking ability (*31*)), but X·F and X·Z pairs are cleaved 24−40 times faster than T·A. However, the correlation is more successful if we use the thermodynamic values obtained for the oligonucleotides with two dangling deoxycytidine nucleotides at the 5′-terminus. This latter context resembles more closely the sequences used in the editing experiments, since the template strand overhangs the end to be edited. In this sequence context the mismatch A·C is 0.9 kcal·mol^{-1} less stable than the correct base pair A·T, and similar values are observed for the nonpolar base pairs A·F and T·Z, which are 0.8 and 0.6 kcal·mol^{-1} less stable, respectively. These values correlate well with the relative orders of editing efficiency.

Carver et al. (*19*) measured partitioning free energies between the polymerase and the exonuclease site for single mismatches at the terminal base pair in the sequence −AAGGX. The average free energy difference between correct and mismatched base pairs was found to be −0.6 to −0.7 kcal·mol^{-1}. When they compared these values with melting data in the blunt-end sequence −ATGGX at the 3′-terminus (*20*), they did not find good correlation due to the small differences in stability (−0.2 kcal·mol^{-1}) between correct and mismatched base pairs. For that reason, they suggested a mixed model wherein weaker DNA binding at the polymerase site and greater melting capacity at the primer terminus both contribute to partitioning of mismatched primer termini into the exonuclease site of the Klenow fragment.

In contrast to that earlier work, we observe a good correlation between our melting data measured with the oligonucleotides having overhanging ends (sequence with similar G·C content, −TCCAX) and the published partitioning free energy difference between correct and mismatched base pairs. We suggest that the use of blunt-ended duplexes as the model can lead to misleading data that are not as relevant to the structural context of the editing complex, and that the overhanging ends are important to overall base pair stabilities. Our data show that both the proofreading rates and the partitioning free energies correlate well with the thermodynamic values in a sequence context more similar to that actually used by the DNA polymerase. Thus, we conclude that the stability of the DNA terminus is the chief determinant of the editing rate. Our data suggest, further, that hydrogen-bonding differences in the terminal pair alone can cause a large difference in 3′-end editing. We observe a large difference between proofreading of A·F and that of A·T (and between proofreading of T·Z and that of T·A), even when T_m values are only different by a small amount. This might be explained by the relative rates of fraying of the terminal pairs; we and others have observed evidence that a non-H-bonded pair may undergo more rapid breathing (*26*, *32*).

Finally, the finding that the rate of proofreading of isostere F is slow relative to that of T raises the possibility that the enzyme may make a hydrogen-bonded contact with bases in the exonuclease cleft. The X-ray structure of the complex of Kf DNA polymerase with a duplex DNA substrate containing a 3′ overhanging single strand shows a 4.8 Å distance between the NH_2 group of the terminal cytosine and Glu474 side chain, which may allow for a water-mediated hydrogen bond (*9*). In addition, a recent crystal structure of the complex of Kf with single-stranded DNA (*33*) shows a shorter distance (3.4 Å) between the O6 of the carbonyl of the terminal guanosine and Glu474. Such a water-bridged interaction may be formed on the major groove side of any natural base. In the case of Z, we observe a rate of proofreading closer to that of the natural bases, which could be due to the possibility of forming a hydrogen bond through its N7 group. It is, however, difficult at present to draw firm conclusions from the direct editing of F and Z, since different factors could be contributing to the slower cleavage relative to that of the natural bases. Further studies will be needed to clarify these effects.

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