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Hydrogen-Bonding Capability of a Templating Difluorotoluene Nucleotide Residue in an RB69 DNA Polymerase Ternary Complex

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Supporting Information

ABSTRACT: Results obtained using 2,4-difluorotoluene nucleobase (dF) as a nonpolar thymine isostere by Kool and colleagues challenged the Watson−Crick dogma that hydrogen bonds between complementary bases are an absolute requirement for accurate DNA replication. Here, we report crystal structure of an RB69 DNA polymerase L561A/S565G/Y567A triple mutant ternary complex with a templating dF opposite dTTP at 1.8 Å-resolution. In this structure, direct hydrogen bonds were observed between: (i) dF and the incoming dTTP, (ii) dF and residue G568 of the polymerase, and (iii) dF and ordered water molecules surrounding the nascent base pair. Therefore, this structure provides evidence that a templating dF can form novel hydrogen bonds with the incoming dTTP and with the enzyme that differ from those formed with a templating dT.

The 2′-deoxyribo-2,4-difluorotoluene nucleobase dF (Figure 1), a reportedly nonpolar, non-hydrogen-bonding isostere of dT,[1] can be recognized by both A and B family DNA polymerases (pols).[2,3] When it is in the form of an incoming deoxynucleoside triphosphate, it is incorporated opposite a templating dA in preference to the other three naturally occurring dNTPs but with lower efficiency than dTTP.[4] When dF is in the templating position, both A and B family DNA polymerases select dATP over the other dNTPs for primer-extension.[5] We have determined the crystal structure of the ternary complex of a mutant RB69 DNA polymerase containing the primer/template duplex and the incoming dTTP opposite dF in the templating position at 1.8 Å-resolution to show that dF has a novel hydrogen bonding capability in the polymerase active site during DNA synthesis.

2,4-Difluorotoluene has been studied in solution when it partitions into the organic phase with a logP value of 0.78 and it is indeed highly nonpolar.[3,6] When dF is part of a DNA duplex opposite dA, it destabilizes the DNA despite its shape similarity to dT.[7] Therefore, it was thought that dF not only fails to hydrogen bond to dA in the complementary strand, but actually results in a net repulsion between dA and dF because dA has a preference for hydrogen-bonding to water.[8] When dF was placed in the middle of a Dickerson-Drew dodecamer DNA duplex opposite dA, which was then bound to BhRNase H, the 1.6 Å-resolution crystal structure of this complex showed that dF exhibited direct hydrogen bond (HB) engagement with dA.[9] However, such dF/dA (or rF/rA[10])-containing structures with dF/dA embedded in the middle of duplexes, not at their ends, do not accurately reflect structural features of the nascent dF/dATP base pair in a pol ternary complex during nucleic acid synthesis. Given what appears to be a conflicting interpretation about the ability of dF to participate in HB interactions, we decided to investigate its behavior when it was the templating partner in a nascent-base-pair binding pocket and a component of a ternary DNA pol:primer/template:dNTP complex. Since dF had not been studied in this context previously, we chose to determine crystal structures of a DNA polymerase ternary complex that met this requirement. This would provide the most direct evidence for its role during DNA synthesis. We designed a L561A/S565G/Y567A triple mutant RB69 pol that incorporated dAMP opposite dF more efficiently than the wild-type (wt) enzyme and it accepted the other three dNTPs as part of the ternary complex where wt RB69pol did not.[10] In addition, the RB69pol triple mutant had the same pre-steady-state kinetic parameters for the incorporation of correct dNMPs as the wt RB69pol.[10] Here, we report the crystal structure of the RB69pol triple mutant ternary complex with dTTP opposite dF at 1.8 Å-resolution. The structure has been refined with a free *R*-factor of 20.3% (Table 1).

An overview of the ternary complex and a close-up of the electron density map for the nascent dTTP/dF base-pair are shown in Figure 2. The electron densities of the templating dF and the surrounding network of ordered water molecules were well-defined. In this structure, dF has a direct wobble HB between F4 of dF and N3−H of dTTP, and another HB between F2 of dF and the Cα−H of G568. The HB distance between N3 of dTTP and F4 of dF is 2.97 Å, and the HB distance between Cα of G568 and F2 of dF is 2.96 Å (Figure 2). The distance between the C3−H of dF and O2 of incoming dTTP is 3.7 Å, which is too great to form a HB. In addition, the C3−H of dF was able to avoid contact with polar groups in such a way as to prevent ordered water molecules from being intercalated at the interface between dTTP and dF. We want to emphasize that the shape and hydrogen bond patterns observed here in the nascent dF/dTTP base pair differ from the dT/dT base pair embedded in the middle of a DNA duplex in complex with a polymerase, where two wobble interbase hydrogen bonds are formed with interatomic distances of 2.91 and 3.34 Å for the O4/N3 and N3/O2 pairs, respectively.[11]

This structure provides direct evidence that a templating dF can form novel HBs that differ from those formed with a templating dT. In the dTTP/dF ternary complex, there are two direct HBs, one between dF and the incoming dTTP and the other between dF and the protein residue G568. In addition, F4 appears to HB

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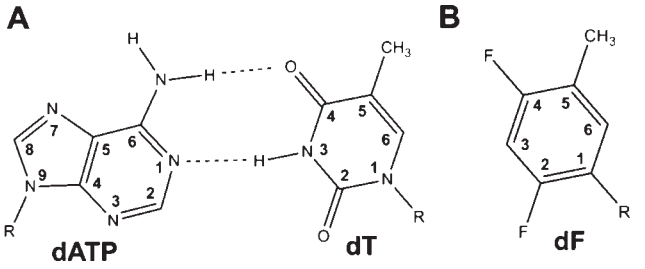

Figure 1. An isostere of dT. (A) Complementary dA/dT base pair; (B) 2,4-difluorotoluene analogue.

Table 1. Crystallographic Statistics for Data Collection and Refinement of the Templating Base dF-Containing Ternary Complex of the RB69pol Triple Mutant

	dTTP opposite dF
Space group	$P2_12_12_1$
Unit cell (*a*, *b*, *c* (Å))	75.13, 119.77, 130.48
Resolution (Å)[a]	50.0−1.80 (1.83−1.80)
No. of unique reflections	101 984
Redundancy	4.2 (2.8)
Completeness (%)	98.4 (87.0)
R_{merge} (%)[b]	9.8 (89.2)
I/σ	14.0 (1.3)
Final model content:	
Amino acid residues	903
Water molecules	1234
Ca^{2+} ions	4
Template nucleotides	18
Primer nucleotides	13
dTTP	1
R (%)[c]	16.7 (29.2)
R_{free} (%)[c]	20.3 (32.6)
rmsd[d]	
Bond length (Å)	0.008
Bond angles (deg)	1.16
R_{o2p}[e]	2.73
PDB accession	3QEP

[a] Statistics for the highest resolution shell are in parentheses. [b] $R_{merge} = \Sigma_{hkl}\Sigma_j|I_j(hkl) - \langle I_j(hkl)\rangle|/\Sigma_{hkl}\Sigma_j\langle I(hkl)\rangle$, statistics for merging all observations for given reflections. [c] $R = \Sigma_{hkl}|F_{obs}(hkl) - F_{calc}(hkl)|/\Sigma_{hkl}F_{obs}(hkl)$, statistics for crystallographic agreement between the measured and model-calculated amplitudes. R_{free} is the agreement for cross-validation data set. [d] Root mean squares deviations (rmsd) to ideal values. [e] The observation-to-parameter ratio (R_{o2p}) is the ratio between the number of unique reflections and the number of parameters, the latter of which is four times the number of atoms (i.e., *x*, *y*, *z*, and B parameters).

to three ordered water molecules with HB distances varying from 2.90 to 3.34 Å. None of these features were anticipated since the chemical properties of dF, determined in the absence of polymerase, indicated that it was nonpolar and unable to participate in HB interactions.[2,3] In addition, we also determined crystal structure of the RB69pol Y567A single mutant with a templating dF opposite dATP at slightly lower resolution (2.3 Å). As shown in Supporting Information Figure 1, the distance between F4 of

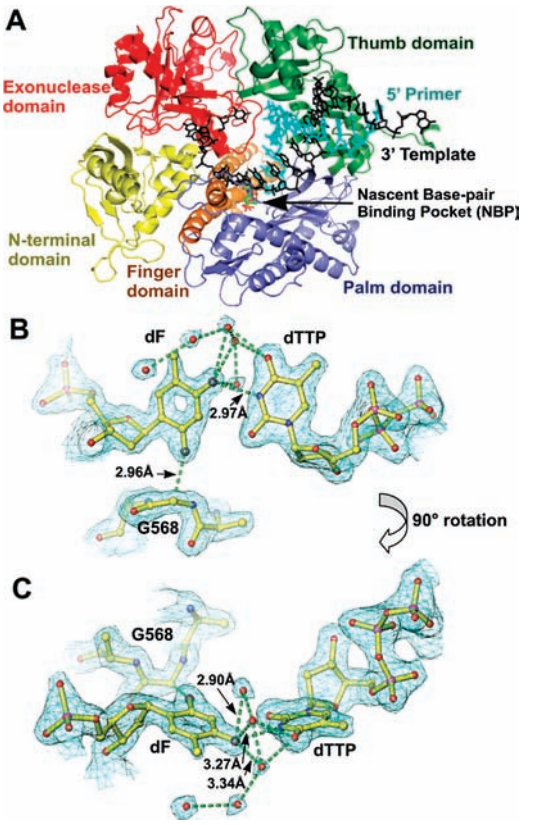

Figure 2. Overall structure of the dF/dTTP-containing ternary complex of the RB69pol triple mutant. (A) The pol structure is colored by domains: the N-terminal domain (residues 1−108 and 340−382) in yellow, exonuclease (residues 109−339) in red, Palm (residues 383−468 and 573−729) in purple, Fingers (residues 469−572) in orange, and Thumb (residues 730−903) in green. The primer and template strand of the DNA are shown in a stick representation, with the template in black and the primer in cyan. (B) A close-up of the nascent base pair superimposed onto the final $2F_o - F_c$ map at 1.8-Å resolution contoured at 1.0σ (cyan chicken wires). (C) An orthogonal view of panel B.

dF and N6−H of incoming dATP is 4.3 Å, which is too great for a HB. Therefore, the direct HB between dF and incoming dTTP observed in the geometrically less-constrained triple mutant is not enforced by the enzyme, nor by the DNA backbone. However, the resolution of this dF/dATP-containing structure is not sufficiently high to observe all ordered water molecules near F4 of the templating dF.

It is worth noting that the overall structure of the RB69 pol triple mutant ternary complex with dTTP opposite dF is almost superimposeable with that of the wt RB69 pol ternary complex with dATP opposite dT except for the nascent base-pair binding pocket, where the residues in the wt pol have been replaced by Ala or Gly.[12] In addition, Gly568 has shifted down toward the interior of the polymerase allowing mispaired dNTPs to be accommodated in the ternary complex. Although the crystal structure reported here clearly demonstrates the partial polar character of a templating dF, it does not provide an explanation for the base selectivity exhibited by dF for dATP, an issue that will be addressed in a subsequent report. Data deposition: refined atomic coordinates and the observed structure factors have been deposited in the Protein Data Bank with PDB access code 3QEP and 3RWU.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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