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DNA Polymerase Selectivity: Sugar Interactions Monitored with High-Fidelity Nucleotides**

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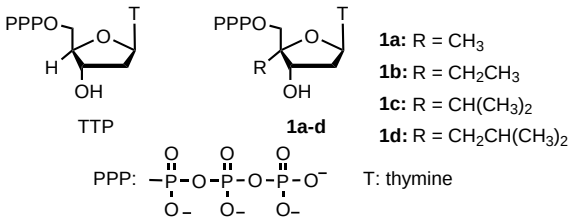
The essential prerequisite of any organism is to keep its genome intact and to accurately duplicate it before cell division. All DNA synthesis required for DNA repair, recombination, and replication depends on the ability of DNA polymerases to recognize the template and correctly insert the complementary nucleotide. In a current model, fidelity is achieved by the ability of DNA polymerases to edit nucleobase pair shape and size.[1] This model is supported by crystal structures of DNA polymerases that suggest the formation of nucleotide binding pockets, which exclusively accommodate Watson–Crick base pairs.[2] Nevertheless, structural data of DNA polymerases complexed with the DNA substrates and a noncanonical triphosphate, which would be very helpful for gaining insights into the causes of error-prone DNA synthesis, have at present not been reported. Thus, functional studies of DNA polymerases with nucleotide analogues have been shown to be extremely valuable.[1] Since most functional studies have focused on nucleobase recognition processes,[1] little is known about the impact of DNA polymerase interactions with the 2′-deoxyribose moiety and the participation of these interactions in processes which contribute to fidelity. Crystal structures of DNA polymerases together with enzyme mutation studies suggest that the sugar moiety of the incoming triphosphate is fully embedded in the nucleotide binding pocket and undergoes essential interactions with the enzyme.[1a, 2] Here we report a functional strategy to monitor steric constraints in DNA polymerases that act on the sugar moiety of an incoming nucleoside triphosphate within the nucleotide binding pocket. We found that novel modified nucleotide probes are substrates for a DNA polymerase with significantly increased selectivity compared to their natural counterpart. Through use of these high-fidelity nucleotides in functional investigations we could show that enzyme–sugar interactions are involved in DNA-polymerase fidelity mechanisms.

In order to sense interactions of DNA polymerases with the sugar moiety of incoming triphosphates we introduced alkyl labels at the 4′-position in the 2′-deoxyribose moiety in such a way that they do not interfere with hydrogen bonding, nucleobase pairing, and stacking. We designed steric probes **1a–d** by substituting the hydrogen atom at the 4′-position of thymidine triphosphate (TTP) with alkyl groups of different size (Scheme 1).



Scheme 1. Thymidine-5′-triphosphate (TTP) and the steric probes **1a–d**.

The synthesis of nucleosides **4a** and **4b** with different synthetic strategies (see Scheme 2) has been reported previously.[3, 4] However, these methods are not suitable for the synthesis of all the targeted compounds and the formation of undesired by-products was observed in the synthesis of **4a**.[3] Thus, we developed a more suitable access to the target compounds. Our synthesis started with the known alcohol **2**, which is easily accessible as described recently.[5] Compound **2** was converted into the methylated thymidine **4a** by functional-group interconversions (Scheme 2). Alkylated nucleosides **4b–d** were synthesized from known compounds[5] by employing Wittig reaction, desilylation, and subsequent reduction of the aliphatic double bond. Next, nucleosides **4a–d** were converted into the desired triphosphates **1a–d** following standard procedures.[6] In order to gain insights into potential effects of the modifications on the sugar conformation we performed conformation analysis based on coupling constants deduced from the ^{1}H NMR data by employing described methods.[7] We found only small differences in **1a–d** compared to natural TTP, which indicates that similar sugar conformations are present in solution (see Supporting Information).

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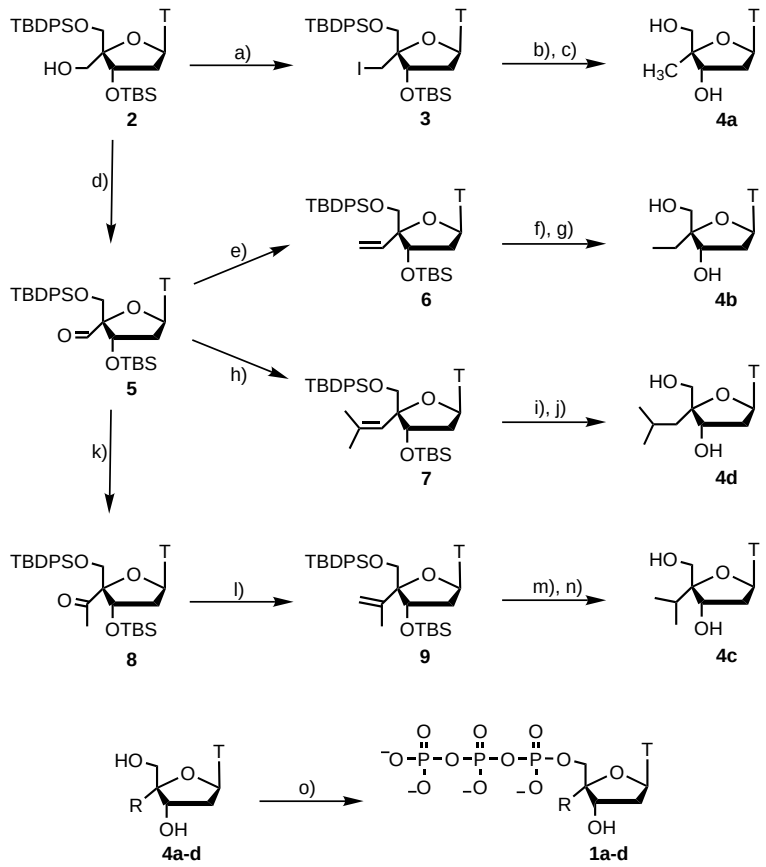

Scheme 2. Syntheses of **1a**–**d**. a) Ph_3P, I_2, imidazole, C_6H_6, 50 °C, 85 %; b) Pd/C, H_2, EtOH, EtOAc, NEt_3; c) TBAF, THF, 79 % (two steps); d) oxidation;[5] e) CH_3PPh_3Br, *n*BuLi, THF, −78 to 25 °C, 99 %; f) TBAF, THF; g) Pd/C, H_2, CH_3OH, 88 % (two steps); h) $(CH_3)_2CHPPh_3I$, *n*BuLi, THF, −78 to 25 °C, 83 %; i) TBAF, THF; j) Pd/C, H_2, CH_3OH, 94 % (two steps); k) alkylation and oxidation;[5] l) CH_3PPh_3Br, *t*BuOK, THF, 25 °C, 91 %; m) TBAF, THF; n) Pd/C, H_2, CH_3OH, 84 % (two steps); o) $POCl_3$, 1,8-bis(dimethylamino)naphthalene, $(CH_3O)_3PO$, 0 °C, then $(nBu_3NH)_2H_2P_2O_7$, nBu_3N, DMF, then 0.1 M aqueous $(Et_3NH)HCO_3$, 23–68 %. TBDPS = *tert*-butyldiphenylsilyl, TBS = *tert*-butyldimethylsilyl, TBAF = tetrabutylammonium fluoride, THF = tetrahydrofuran, DMF = *N*,*N*-dimethylformamide.

We tested the effect of probes **1a**–**d** on the Klenow fragment (KF^-) of *E. coli* DNA polymerase I (*exo*$^-$-mutant), an enzyme extensively used as a model for investigations into intrinsic DNA-polymerase mechanisms and function.[1] We used a gel-based single nucleotide insertion assay,[8] where an adenine in the template strand codes for the insertion of a thymidine analogue adjacent at the primer 3′-end,[9] to monitor polymerase function. Figure 1 A shows the pattern of insertion of thymidine-5′-triphosphate (TTP) and **1a**–**d** catalyzed by KF^-. The results obtained reveal that **1a**–**d** are functionally competent, although with varied efficiencies.

In order to quantify these observations we determined the insertion efficiencies (v_{max}/K_M; v_{max} = the maximum rate of the enzyme reaction, K_M = the Michaelis constant) of the thymidine analogues by investigation of single-nucleotide insertion at various nucleotide concentrations under single completed hit and steady-state conditions as recently described.[8, 10–12] The quantitative analyses revealed that KF^- incorporates **1a** and **1b** with virtually the same efficiency as unmodified TTP (Table 1). Thus,

Table 1. Steady-state analyses for canonical nucleoside triphosphate insertion. The data presented are averages of duplicate or triplicate experiments. Further experimental details are described in Supporting Information.

Nucleoside triphosphate	K_M [μM]	v_{max} [$min^{-1} \times 10^{-3}$]	v_{max}/K_M [$M^{-1} min^{-1}$]
TTP	0.11 ± 0.05	20 ± 3	180000
1a	0.40 ± 0.01	58 ± 4	150000
1b	0.21 ± 0.01	29 ± 5	140000
1c	47 ± 4	85 ± 5	1800
1d	241 ± 22	76 ± 1	320

it appears that 4′-methyl and -ethyl groups are nicely accommodated within the nucleotide binding pocket of KF^-. Further increase in the bulk of the probes caused a marked decrease in the insertion efficiency of **1c** and **1d**, presumably due to a steric clash since the modifications do not cause significant perturbation of the sugar pucker conformation.

Since the ratio of nucleotide-insertion efficiencies opposite a complementary to a noncomplementary nucleobase is a measurement of DNA-polymerase fidelity,[8, 10, 11] we next investigated the ability of KF^- to catalyze non-Watson–Crick nucleobase pair formation. We focused on **1a** and **1b**, since only these are inserted as efficiently as TTP by KF^- in

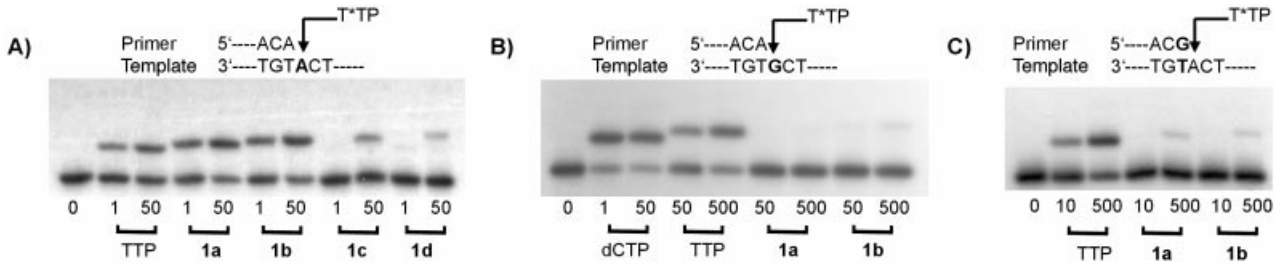

Figure 1. Nucleoside triphosphate insertion catalyzed by KF^-. A) Insertion encoded by adenine (A) in the template strand. Conditions: Primer/template complex (50 nM), KF^- (2 nM), 37 °C, 5 min. B) Nucleoside triphosphate insertion encoded by guanine (G) in the template strand. Conditions: Primer/template complex (50 nM), KF^- (4 nM), 37 °C, 5 min. C) Mismatch extension. Conditions: Primer/template complex (50 nM), KF^- (2 nM), 37 °C, 20 min. In each case, the nucleotide concentrations [μM] are shown in the figure. dCTP = 2′-deoxycytidine-5′-triphosphate, T*TP = TTP analogues. Further experimental details and the DNA sequences employed are described in the Supporting Information.

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canonical base pair formation and thus they should be ideally suited for use in the monitoring of differential enzyme interactions between insertion and misinsertion events. Assays of nucleotide incorporation opposite G, T, and C revealed that KF$^-$ misinserts TTP and **1a**, **b** opposite G with the highest efficiency.[9, 13] Strikingly, as is already apparent from the results shown in Figure 1B, **1a** and **1b** exhibit dramatically decreased misinsertion efficiency. Steady-state kinetic analyses revealed an approximately 100-fold decrease in misinsertion efficiency using the 4′-alkylated probes **1a**, **b** compared to TTP (Table 2).

Table 2. Steady-state analyses for nucleoside triphosphate misinsertion and mismatch extension. The data presented are averages of duplicate or triplicate experiments. Further experimental details are described in Supporting Information.

Nucleoside triphosphate	K_M [μM]	v_{max} [$min^{-1} \times 10^{-3}$]	v_{max}/K_M [$M^{-1} min^{-1}$]
misinsertion:			
TTP	22 ± 0.2	16 ± 1	730
1a	65 ± 5	0.34 ± 0.02	5
1b	228 ± 5	1.7 ± 0.1	7
mismatch extension:			
TTP	19 ± 1	36 ± 2	1900
1a	80 ± 17	2.9 ± 0.1	40
1b	40 ± 5	4.0 ± 0.3	100

Further experiments also showed that the analogues **1a** and **1b** were not as well inserted opposite T or C as unmodified TTP was (see Supporting Information). These results clearly show that nucleotide-insertion selectivity is increased by substitution of the hydrogen atom at the 4′-position of the sugar with bulkier alkyl groups.

The second critical determinant of intrinsic DNA-polymerase fidelity is the capacity to extend from mismatched primer/template ends.[1a, 8, 11] Here again, **1a** and **1b** should be ideally suited to monitor differential DNA-polymerase interactions with the sugar moiety in mismatch extension events. We investigated extension from template-T/primer-G, T/T, and T/C by using KF$^-$ to extend from a mismatched base at the primer 3′-end by incorporation of TTP or **1a**, **b**. The most efficient extension was from T/G for all thymidine triphosphate derivatives (data shown for T/G in Figure 1C).[9, 13] The results of quantitative analyses of T/G extensions are presented in Table 2. KF$^-$ extends a T/G mismatched primer end with probes **1a**, **b** significantly less efficiently than with TTP. Again, this is in contrast to the results in Table 1 where TTP and **1a**, **b** extension of a perfectly matched primer were equivalent. Our results indicate that nucleobase pair mismatches at the 3′-end of a primer/template complex trigger unfavorable enzyme interactions with the sugar moiety of an incoming nucleoside triphosphate and prevent inadvertent sealing of a base substitution in the nascent DNA strand. Indeed, this mechanism may be considered essential for proofreading, and may allow a pause for other functions such as intrinsic exonuclease activity or dissociation of the DNA-replication complex.

In conclusion, the selectivity of nucleotide insertion by a DNA polymerase can be significantly increased by modified sugar moieties. Our results strongly implicate the involvement of differential DNA-polymerase interactions with the sugar in processes that contribute to the fidelity of DNA synthesis. Furthermore, our studies provide a new functional and general method to monitor steric constraints in nucleotide binding pockets of DNA polymerases. Further analyses with such steric probes, both at the functional and structural level should reveal more insights into mechanisms of DNA polymerase selectivity.

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