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Varied Active-Site Constraints in the Klenow Fragment of *E. coli* DNA Polymerase I and the Lesion-Bypass Dbh DNA Polymerase

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We report on comparative pre-steady-state kinetic analyses of exonuclease-deficient Escherichia coli *DNA polymerase I (Klenow fragment, KF⁻) and the archaeal Y-family DinB homologue (Dbh) of* Sulfolobus solfataricus*. We used size-augmented sugar-modified thymidine-5′-triphosphate ($T^{R}TP$) analogues to test the effects of steric constraints in the active sites of the polymerases. These nucleotides serve as models for study of DNA polymerases exhibiting both relatively high and low intrinsic selectivity. Substitution of a hydrogen atom at the 4′-position in the nucleotide analogue by a methyl group reduces the maximum rate of nucleotide incorporation by about 40-fold for KF⁻ and about twelvefold for Dbh. Increasing the size to an ethyl group leads to a further twofold reduction in the rates of incorporation for both enzymes. Interestingly, the affinity of KF⁻ for the modified nucleotides is only marginally affected, which would indicate no discrimination during the binding step. Dbh even has a higher affinity for the modified analogues than it does for the natural substrate. Misincorporation of either TTP or $T^{Me}TP$ opposite a G template causes a drastic decline in incorporation rates for both enzymes. At the same time, the binding affinities of KF⁻ for these nucleotides drop by about 16- and fourfold, respectively, whereas Dbh shows only a twofold reduction. Available structural data for ternary complexes of relevant DNA polymerases indicate that both enzymes make close contacts with the sugar moiety of the dNTP. Thus, the varied proficiencies of the two enzymes in processing the size-augmented probes indicate varied flexibility of the enzymes' active sites and support the notion of active site tightness being a criterion for DNA polymerase selectivity.*

Introduction

The accurate replication of DNA is essential for all forms of life. This process is performed by replicative DNA polymerases with errors rates as low as 10^{-6} (in the absence of proofreading).[1–3] However, recently discovered DNA polymerases involved in lesion bypass synthesis exhibit strikingly low fidelity when dealing with undamaged DNA.[4] The fidelity is somewhat improved when dealing with certain forms of damaged DNA.[5,6] An understanding of the basis for the differences in fidelity between polymerases involved in lesion bypass and in replication is essential to understand the process of DNA replication.

Various models to account for the high selectivity of DNA polymerases during DNA replication have been suggested. At first glance the formation of distinct hydrogen-bonding patterns between the nucleobases of the coding template strand and the incoming nucleoside triphosphate appears to be responsible for accurate information transfer. Yet, as suggested by Echols and Goodman on the basis of thermal denaturating studies of matched and mismatched DNA complexes, these interactions alone are not sufficient to explain the degree of accuracy commonly observed for enzymatic DNA synthesis.[2] Several additional factors have been suggested to be involved in correct nucleotide recognition. Among these factors are exclusion of water from the enzyme's active site, base stacking, solvation, minor groove scanning and steric constraints within the nucleotide binding pocket.[7] In the context of an attempt to evaluate the contribution of hydrogen bonding to DNA polymerase fidelity, Kool et al.[7] described a functional strategy based on chemically modified nucleotide substrates bearing nonpolar aromatic molecules that closely mimicked the shapes and sizes of the natural nucleobases but showed significantly diminished ability to form stable hydrogen bonds. These nonpolar nucleotide isosters were applied as functional probes to elucidate the effect of hydrogen bonding on DNA polymerase selectivity. Kool and co-worker found that the nonpolar isosters were processed by several DNA polymerases with remarkably high selectivity and efficiency.[7] From these results it was concluded that hydrogen bonding is not absolutely essential in order to achieve high incorporation efficiencies and that significant levels of selectivity can be achieved without it. Close fitting of Watson–Crick geometry along with specific minor groove interactions are among the most important factors for achieving selectivity in DNA replication.

Several crystal structures of DNA polymerases in complex with their DNA and dNTP substrates have contributed signifi-

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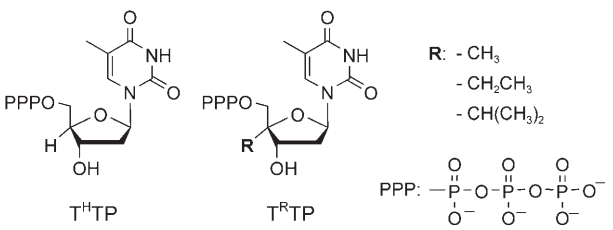
cantly to our understanding of substrate recognition by these enzymes.[8–12] The structures of DNA polymerases have been likened to a right hand consisting of fingers, palm and thumb subdomains, forming a large cleft that binds the primer/template. The palm domain harbours the catalytic centre containing the essential carboxylates involved in the phosphoryl transfer reaction. The high degree of conservation of this domain throughout distinct DNA polymerase families from eukaryotic, prokaryotic and viral DNA polymerases is striking.[5] In contrast, the thumb and finger domains, which show extensive contacts with the primer/template complex and with the incoming dNTPs, respectively, differ significantly among DNA polymerases.

Numerous biochemical studies of DNA polymerase reaction pathways have led to the establishment of a minimal kinetic model for nucleotide incorporation.[13–23] It is believed that the reaction follows an ordered series of distinguishable microscopic events. Firstly, the enzyme binds to the primer/template complex, and this is followed by binding of the incoming dNTP. Nucleotide binding then triggers the formation of an activated complex, after which the chemical bond is formed. Formation of the activated complex is believed to be the rate-limiting step of nucleotide incorporation. Several studies strongly support the occurrence of large conformational changes from an "open" to a "closed" conformation prior to phosphodiester bond formation triggered through dNTP binding.[3,8,9,11,24,25] Whether the open-to-closed transition due to dNTP binding is the rate-limiting step is still a matter of debate,[26–28] although this substrate-induced change in the position of the fingers domain is believed to be of major importance for nucleotide selection.[29] Crystallographic data for DNA polymerases indicate that these enzymes adopt conformations that preferentially accommodate the geometry of Watson–Crick base pairs. This might be a further indication that geometrical constraints are at least one cause of DNA polymerase fidelity.

Endo- and exogenous stress (for example, reactive oxygen species, chemicals, radioactivity, ultraviolet (UV) radiation etc.) cause DNA damage, necessitating specifically adapted strategies in order to repair such lesions.[30] UV light, for example, causes a variety of forms of damage to DNA. The most abundant lesions are pyrimidine dimers such as the pyrimidine pyrimidone photoproduct (6–4PP) and the *cis-syn* cyclobutane pyrimidine dimer (CPD). Often these lesions are repaired sluggishly and remain in the DNA, causing considerable impairment and eventually stalling of the DNA replication machinery. How cells perform DNA synthesis past these kind of lesions has long been obscure, and only recently has it been discovered that several specialized DNA polymerases belonging to the new Y-family are involved in translesion synthesis.[5,31,32] One of their most prominent functional characteristics is their high error propensity when dealing with undamaged DNA, which distinguishes them from known high-fidelity DNA polymerases (for a recent review see Yang and Woodgate[33]).

The individual selectivities of DNA polymerases may vary by up to several orders of magnitude. However, the underlying mechanism for these variations is only sparsely understood. Insights into error-prone DNA synthesis have recently been gained with the help of crystal structures of several Y-family polymerases, such as the N-terminal catalytic domain of yeast DNA polymerase η and *Solfolobus solfataricus* (P1 and P2, respectively) DNA polymerases Dbh (DinB homologue) and Dpo4 (DNA polymerase IV); in particular, the latter (DNA polymerase IV) crystallized in a ternary complex with DNA and an incoming nucleotide.[34–37] Moreover, important fundamental insights into structural differences between different DNA polymerases were gained by comparison of these crystal structures with those from high-fidelity enzymes. In brief, the structures of the low-fidelity enzymes indicate that the nascent base pair between the template and the incoming nucleotide is less tightly coordinated than it would be in a high-fidelity DNA polymerase. A solvent-accessible active site has been suggested as an important structural feature for the error-prone replication of DNA by this class of DNA polymerases. Interestingly, another structure of the error-prone DNA polymerase Dpo4 showed a noncanonical dNTP bound to the active site.[37] From this structure it is apparent that conformations both of the active site amino acids and of the sugar phosphate moieties of the primer, template and nucleoside triphosphate in the active site differ significantly from those observed when a canonical nucleotide is bound. This is due to a translocation of the first template base (G) so that the incoming ddGTP forms a canonical base pair with the next template base (C). Such an alignment might be one reason for the apparent faulty DNA synthesis by this kind of enzymes. Despite that, recent studies performed with nucleotide analogues possessing nonpolar nucleobase surrogates and size variation in sub-ångstrom increments indicate that lesion-bypass Dpo4 DNA polymerase has an active site that is more flexible than those in the more selective enzymes in tolerating size deviations.[38]

In an attempt to unravel some of the fundamentals relating to the different active site constraints of DNA polymerases with and without lesion-bypass ability we compared exonuclease-deficient *Escherichia coli* DNA polymerase I (Klenow fragment, KF^-) and the archaeal Y-family DinB homologue (Dbh) of *Sulfolobus solfataricus* as model systems by applying size-augmented thymidine-5′-triphosphate (TTP) analogues as steric probes. In these TTP analogues (T^RTP) the 4′-hydrogen of the sugar is substituted with alkyl groups (–CH_3, –CH_2CH_3 and –$CH(CH_3)_2$), with gradual expansion of their steric demand (Scheme 1).[39,40] We conducted pre-steady-state kinetic measurements in order to obtain information about nucleotide binding and incorporation, and by these means we were able to derive experimental evidence for varied active site constraints of these two polymerases.



Scheme 1. Thymidine-5′-triphosphate (TTP) and the steric probes.

Results

Time course of single-turnover, single-nucleotide incorporation

We first analysed the single-turnover, single-nucleotide incorporation kinetics of $T^{R}TP$ into a 24/36 nt primer/template (p/t) by KF^{-} and Dbh, respectively. In order to ensure that the observed single-turnover rate of incorporation is limited by internal rate-limiting kinetic parameters, rather than by binding parameters, which occurs when concentrations below the saturation level are used, we carefully examined binding affinities of the incoming dNTP (see section below). All experiments were, if possible, carried out under saturating concentrations of p/t and nucleotide (for details see corresponding Figure legends and Table 1).

In Figure 1 A the time courses of $T^{R}TP$ incorporation by KF^{-} are illustrated in a comparative manner. In agreement with earlier findings by Dahlberg et al.,[16] we observed a biphasic burst of product formation for incorporation of $T^{H}TP$ by the polymerase. Fitting of the experimental data to a double-exponential equation yielded burst rates (k_{pol1} and k_{pol2}) of $(230\pm13)\ s^{-1}$ and $(1.6\pm0.3)\ s^{-1}$. Analysis of the experimental data for $T^{Me}TP$ incorporation with a double-exponential equation gave rates of $(5.7\pm0.4)\ s^{-1}$ for k_{pol1} and $(0.94\pm0.18)\ s^{-1}$ for k_{pol2}. The incorporation of $T^{Me}TP$ had thus resulted in a clear drop (40-fold) in the fast burst rate whereas the slower rate was not affected. A further increase in the size of the alkyl substituent to ethyl yielded an incorporation rate of $(3.2\pm0.1\ s^{-1})$, which was only slightly reduced in relation to the k_{pol1} value in the case of $T^{Me}TP$. However, no second burst phase could be observed for this nucleotide. A further increase in the steric demand of the nucleotide substrate through the usage of $T^{iPr}TP$ caused a more significantly pronounced drop in the incorporation rate in relation to the unmodified substrate $T^{H}TP$ (11 000-fold).

Unlike in the case of KF^{-}, incorporation of $T^{H}TP$ by the Dbh bypass polymerase occurs in a monophasic burst of product formation (Figure 1 B) with a rather slow incorporation rate of $(0.6\pm0.03)\ s^{-1}$, as recently described.[23] The incorporation of $T^{Me}TP$ resulted in a twelvefold reduction in the rate down to $(0.05\pm0.002)\ s^{-1}$. An increase in the size of the alkyl substituent to ethyl resulted in a slightly reduced rate of $(0.02\pm0.001)\ s^{-1}$. Unlike in the KF^{-} case, $T^{iPr}TP$ proved to be a poor substrate for Dbh, so we were unable to measure any incorporation rate faithfully.

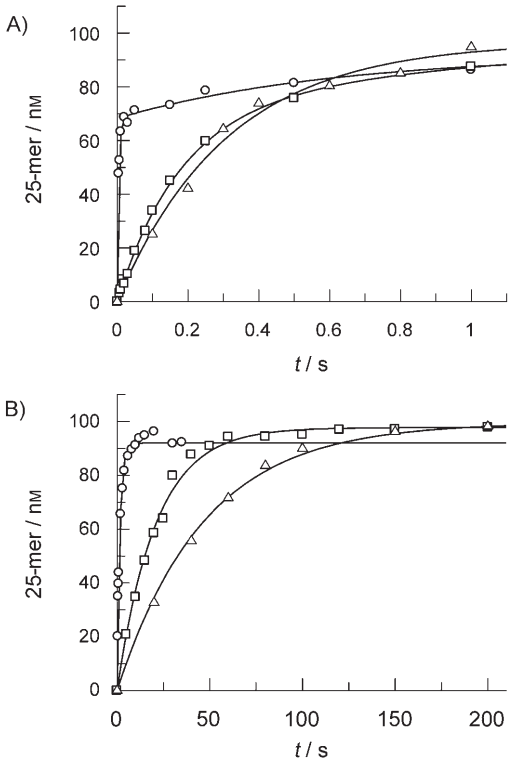


Figure 1. Single-turnover, single-$T^{H}TP$, -$T^{Me}TP$ or -$T^{Et}TP$ incorporation into 24/36 DNA/DNA p/t by KF^{-} and Dbh. The curves show the best fit of the data to a double or single exponential equation. A preformed complex of 200 nM KF^{-} (A) or 1.5 μM Dbh (B) and 100 nM p/t was rapidly mixed with 400 μM $T^{H}TP$ (○), 600 μM $T^{Me}TP$ (□) or 500 μM $T^{Et}TP$ (△) in the case of KF^{-} (A), or with 3 mM $T^{H}TP$ (○), 0.8 mM $T^{Me}TP$ (□) and 0.7 mM $T^{Et}TP$ (△) in that of Dbh (B). The analysis of the KF^{-} data yielded two burst rates (k_{pol1} and k_{pol2}) of $(230\pm13)\ s^{-1}$ and $(1.6\pm0.3)\ s^{-1}$ for the incorporation of $T^{H}TP$ and $(5.7\pm0.4)\ s^{-1}$ and $(0.94\pm0.18)\ s^{-1}$ for the incorporation of $T^{Me}TP$. The relative distributions of these two burst amplitudes were 68 nM and 24 nM for TTP, corresponding to the fast and the slow rates, respectively, and 70 nM and 29 nM for $T^{Me}TP$. A single-exponential analysis was applied for the incorporation of $T^{Et}TP$ and yielded a k_{pol} value of $(3.2\pm0.1)\ s^{-1}$. The best fit of the experimental data for Dbh to a single-exponential equation resulted in incorporation rates of $(0.6\pm0.02)\ s^{-1}$ for $T^{H}TP$, $(0.05\pm0.002)\ s^{-1}$ for $T^{Me}TP$ and $(0.02\pm0.004)\ s^{-1}$ for $T^{Et}TP$.

Table 1. Summary of the pre-steady-state parameters for modified nucleotide incorporation and misinsertion.

$T^{R}TP$	K_d $T^{R}TP$ [μM]		k_{pol} [s^{-1}]		Incorporation efficiency[a] [$\mu M^{-1}s^{-1}$]		Relative incorporation efficiency[b]		RIE KF^{-}/ Dbh	Selectivity factor[c]	
	KF^{-}	Dbh	KF^{-}	Dbh	KF^{-}	Dbh	KF^{-}	Dbh		KF^{-}	Dbh
Opposite A:											
H	33±1.0	590±55	230±13	0.6±0.02	7	1×10^{-3}	1	1	–	–	–
Me	44±6.0	175±10	5.7±0.4	0.05±0.002[d]	1.3×10^{-1}	3×10^{-4}	1.8×10^{-2}	3×10^{-1}	0.06	54	3
Et	53±4.9	330±21	3.2±0.1	0.02±0.004[d]	6×10^{-2}	6×10^{-5}	8.6×10^{-3}	6×10^{-2}	0.15	117	17
*i*Pr	36±7.3		0.02±0.001		5.5×10^{-4}		8.0×10^{-5}			12 700	
Opposite G:											
H	540±30	1300±100	0.02±0.001	0.002±0.0001[d]	4×10^{-5}	1.5×10^{-6}	5.7×10^{-6}	1.5×10^{-3}	0.004		
Me	190±15		0.0005±0.0001	0.0007±0.0001[d]	3×10^{-6}		4×10^{-7}				

[a] Incorporation efficiency (k_{pol}/K_d). [b] Relative incorporation efficiency (RIE) $[(k_{pol}/K_d)_{T^RTP}]/[(k_{pol}/K_d)_{T^HTP}]$. [c] Calculated as $[(k_{pol}/K_d)_{TTP}/(k_{pol}/K_d)_{analogue}]$. [d] Not measured under saturating nucleotide concentrations due to substrate inhibition.

T^RTP binding affinity for correct nucleotide insertion

We next examined the binding affinities of both enzymes for each T^RTP nucleotide used in this study, by measuring the dependence of the pre-steady-state burst rate on the T^RTPs' concentrations (Figure 2). The best fit to a hyperbolic equation relating the rate of incorporation to the nucleotide concentration yielded T^HTP dissociation constants (K_d values) of (33 ± 1.0) μM and (590 ± 55) μM for KF^- and Dbh, respectively, consistently with previous measurements.[13,23,41,42] Analysis of the binding

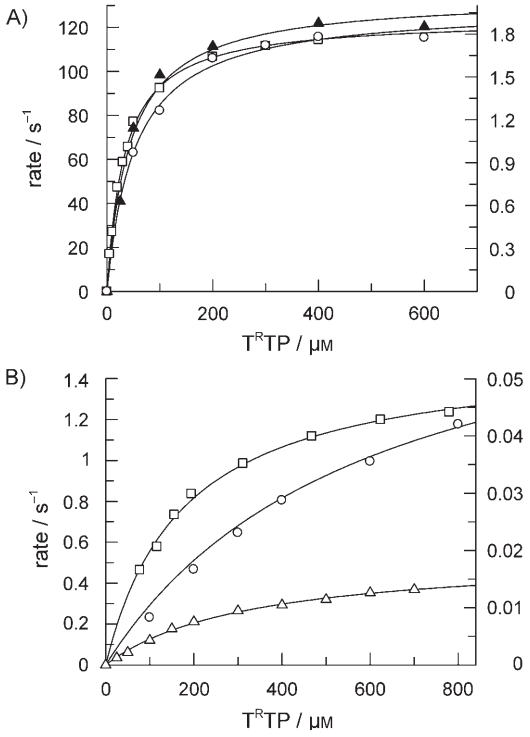


Figure 2. Dependence of the pre-steady-state burst rate on the T^HTP (○), $T^{Me}TP$ (□) or $T^{Et}TP$ (△) concentration. Increasing amounts of T^RTP were rapidly mixed with a preformed complex either of 200 nM KF^- (A) or of 1.5 μM Dbh (B) and 100 nM p/t. Reactions were quenched after $\tau_{1/2}$ of the maximal pre-steady-state rate (see the Experimental Section). Data were fitted to a hyperbolic equation, yielding K_d values for KF^- of (33 ± 1) μM for T^HTP, (44 ± 6.0) μM for $T^{Me}TP$ and (53 ± 4.9) μM for $T^{Et}TP$, and for Dbh of (590 ± 55) μM for T^HTP, (175 ± 10) μM for $T^{Me}TP$ and (330 ± 21) μM for $T^{Et}TP$. The observed rates given on the left *y*-axis correspond to T^HTP and the ones on the right to $T^{Me}TP$ and $T^{Et}TP$.

affinities for $T^{Me}TP$ resulted in K_d values of (44 ± 6.0) μM and (175 ± 10) μM for KF^- and Dbh, respectively. A further increase in the size of the nucleotide through the introduction of an ethyl group at the sugar ring resulted only in a slight change in binding affinity, with a K_d of (53 ± 4.9) μM in the case of KF^- and a K_d of (330 ± 21) μM in that of the Dbh polymerase. Finally, the $T^{iPr}TP$ analogue was shown to bind KF^- with affinities similar to those of the unmodified T^HTP (Table 1). As described above, Dbh does not incorporate $T^{iPr}TP$, so we were unable to measure the binding affinity of the Y-family polymerase for this nucleotide analogue by the technique applied in this study.

Single-turnover nucleotide misincorporation of T^RTP opposite a G template

In a next step we analysed the misincorporation of T^RTP opposite a G template by the two DNA polymerases in order to gain insights into whether the size expansion by 4′-alkylation has an effect on fidelity. As described above, all experiments were performed under saturating nucleotide concentrations if possible. Since, in the case of Dbh, the misincorporation rates were too slow to be measured with the quenched flow apparatus, experiments were performed manually. Figure 3 shows the time courses of misincorporation of T^HTP and $T^{Me}TP$ either by KF^- or by Dbh. The curves show the best fit of the experimental data to a single-exponential equation. For KF^- we determined burst rates of (0.02 ± 0.001) s^{-1} for T^HTP and (0.0005 ± 0.0001) s^{-1} for $T^{Me}TP$. The Dbh bypass polymerase showed a rate of (0.002 ± 0.0001) s^{-1} for the misincorporation of T^HTP. However, we observed extension of less than 30% of the substrate by one nucleotide—relative to correct nucleotide incorporation—which was suggestive of nonproductive enzyme–substrate complexes. This effect was even more pronounced when $T^{Me}TP$ was used. Here, only about 10% of the

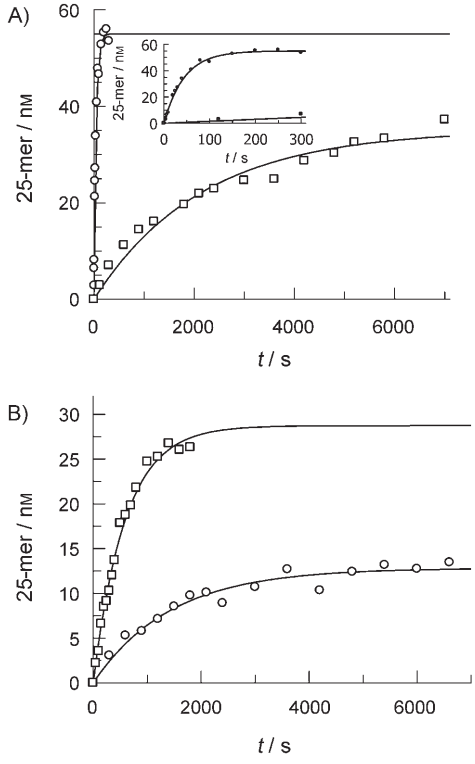


Figure 3. Single-turnover kinetics of misincorporation of T^HTP and $T^{Me}TP$ opposite a G template into 24/36 DNA/DNA p/t by KF^- and Dbh. A preformed complex of 100 nM p/t and 200 nM KF^- (A) or 1.5 μM Dbh (B) was rapidly mixed with T^HTP (○) or $T^{Me}TP$ (□) and quenched at the time points indicated. Preferably to ensure saturating dNTP concentrations, 3 mM T^HTP and 2 mM $T^{Me}TP$ (KF^-) or 4 mM T^HTP and 4 mM $T^{Me}TP$ (Dbh) were used. The solid lines show the best fits of the data by use of a single-exponential equation. Analysis of the experimental data yielded burst rates of (0.02 ± 0.001) s^{-1} and (0.0005 ± 0.0001) s^{-1} for T^HTP and $T^{Me}TP$ (KF^-). In the case of Dbh, rates of (0.002 ± 0.0001) s^{-1} and (0.0007 ± 0.0001) s^{-1} for T^HTP and $T^{Me}TP$ were obtained. The inset shows the reaction on a shorter timescale.

substrate was extended, with a rate of ($0.0011 \pm 0.0002\ s^{-1}$). Interestingly, in the case of the Dbh polymerase the misincorporation rates of $T^{H}TP$ and $T^{Me}TP$ differ only marginally.

$T^{R}TP$ binding affinity for incorrect nucleotide insertion

Analogously to the experiments described above, we also determined nucleotide binding affinities for the misincorporation of $T^{R}TP$ opposite a G template (Figure 4). For KF^{-} the best fit of the experimental data to a hyperbolic equation yielded K_ds of (540 ± 30) μM for $T^{H}TP$ and (190 ± 15) μM for $T^{Me}TP$. For Dbh we were able to derive a K_d value of (1300 ± 100) μM when misincorporating $T^{H}TP$ opposite a G template. Unfortunately we were not able to determine an accurate binding constant for the Dbh/$T^{Me}TP$ interaction in the situation of non-Watson–Crick base pairing; this was due to a drastic reduction in product formation as described above. As a result, the signal dropped below the detection limit of the denaturating PAGE analysis, making a quantitative analysis unfeasible. However, the finding that the maximum observable rate of $T^{Me}TP$ misincorporation did not further increase even on raising the concentration of the nucleotide beyond 4 mM (compare Figure 3) indicates that the measurements were indeed performed under saturating nucleotide concentrations (data not shown). Therefore it can be concluded that the K_d value for $T^{Me}TP$ is at least in the same range as for $T^{H}TP$.

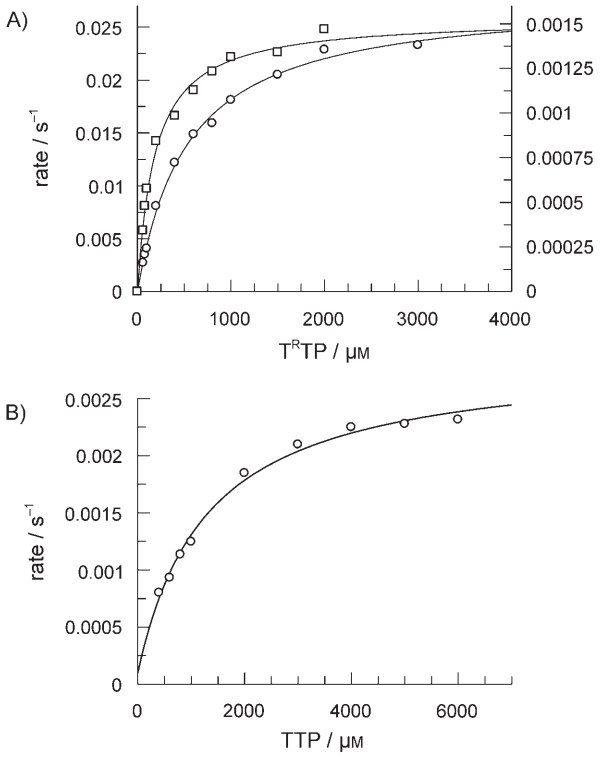


Figure 4. Dependence of the pre-steady-state rate of misincorporation of $T^{R}TP$ opposite a G template on the $T^{R}TP$ concentration. Increasing amounts of $T^{R}TP$ were rapidly mixed with preformed complexes of either A) 200 nM KF^{-} or B) 1.5 μM Dbh and 100 nM p/t. Reactions were quenched after $\tau_{1/2}$ of the maximal pre-steady-state rate (see Figure 2). Data were fitted to a hyperbolic equation, yielding K_d values for KF^{-} of (540 ± 30) μM for $T^{H}TP$ and (190 ± 15) μM for $T^{Me}TP$ and for Dbh of (1300 ± 100) μM for $T^{H}TP$. The observed rates given on the left *y*-axis correspond to $T^{H}TP$ (○) and the one on the right to $T^{Me}TP$ (□).

Discussion and Conclusion

In this study we examined the role of active site tightness on the DNA polymerase fidelity of the exonuclease-deficient *E. coli* DNA polymerase I (KF^{-}) and the archaeal Y-family DinB homologue (Dbh) of *Sulfolobus solfataricus* with the help of steric nucleotide probes ($T^{R}TP$) exhibiting varying steric demand due to the presence of size-augmented sugar residues without alteration of hydrogen bonding capability, by performing pre-steady-state kinetic measurements for the first time (for a summary see Table 1). If the hypothesis of active site tightness as a major factor for nucleotide discrimination holds true, such nucleotide analogues should be an indicator, as has already been shown for HIV-1 reverse transcriptase (RT).[43,44] One could expect that size-augmented nucleotides would be better processed in the incorporation step by a more promiscuous DNA polymerase than by the more selective counterparts.

The substitution of the hydrogen at the 4′-position of the nucleotide sugar ring with a methyl group led to an approximately 40-fold decreased pre-steady-state incorporation rate in the case of KF^{-}, while the incorporation of $T^{Et}TP$ by this enzyme showed only an approximately 1.8-fold further decline in the incorporation rate. The bulkiest $T^{iPr}TP$ is incorporated with a 11 000-fold drop in rate in relation to the unmodified counterpart. Methyl and ethyl groups are thus relatively well tolerated, whereas any further increase in steric demand results in a significant reduction in the observed incorporation rates. On the other hand, the effects of the modifications on nucleotide-binding affinities are only marginal. Even the bulkiest probe—$T^{iPr}TP$—showed a binding affinity for KF^{-} as high as the natural nucleotide. Remarkably, we observed a biphasic burst of nucleotide incorporation by KF^{-}, although, under the experimental conditions chosen, one would expect a single burst of product formation, as was the case with Dbh. At present we can only speculate about the underlying mechanisms. Then again, we have observed similar behaviour for HIV-1 RT. This was eventually interpreted on the basis of two different RT–p/t complexes: a productive enzyme/substrate complex capable of nucleotide incorporation and a nonproductive complex that has to undergo an isomerisation before dNTP incorporation can occur.[20,45] In this context it is interesting to note that Rothwell and Waksman have recently identified two Klentaq1/nucleic acid substrate complexes,[46] so it is quite feasible that KF^{-} shows similar behaviour.

Overall, the findings described above recall at least to some extent what was observed with HIV-1 RT when analogous studies were performed.[43] However, there are striking differences between the two polymerases. RT also incorporates $T^{Me}TP$ and $T^{Et}TP$ with diminished rates in relation to $T^{H}TP$, although the reduction is more pronounced, with about a 180-fold initial drop, while the binding affinities for the modified nucleotides are only slightly affected (less than a factor of two). For $T^{iPr}TP$ the situation is different. Here the incorporation is only about

3000 times slower, while the binding affinity drops by ca. 30-fold. In total, RT is about seven times more stringent than KF^- in discriminating against the incorporation of $T^{iPr}TP$. Interestingly, a similar difference in discrimination is observed for the other two analogues, although the underlying mechanisms are different. This clearly indicates different steric constraints during the progression of nucleotide incorporation from initial binding to the chemical step of these two DNA polymerases.

The Dbh, on the other hand, showed just a twelvefold reduction in the transient $T^{Me}TP$ incorporation rate relative to $T^{H}TP$. A further increase in the size of the steric probe to $T^{Et}TP$ resulted only in an additional twofold drop in the incorporation rate. Analysing the affinities of the different $T^{R}TP$ analogues for Dbh, we did not observe any decrease in binding. Quite the opposite: there was even an increase in affinity for $T^{Me}TP$ and $T^{Et}TP$. This finding could imply that the analogues fit better into the active site because of their expanded sizes and are probably able to make more stabilizing hydrophobic interactions within the nucleotide binding pocket. Thus, the proficiency of this enzyme for accommodation of sterically more demanding nucleotides supports the notion of a looser and more flexible active site of the enzyme.

It has been suggested that because of their larger, sterically more "open" active sites, Y-family DNA polymerases such as the Dbh polymerase are competent in processing size-altered nucleotide pairs.[47] One other model suggests that these enzymes surround the nascent base pair closely but are more flexible, in order to accommodate larger base pairs.[38] It was discussed on the basis of the crystal structure of a ternary complex of the related Dpo4 bypass DNA polymerase that only a few polar contacts of the enzyme to the bound nucleotide can be assigned, in contrast to DNA polymerases exhibiting higher fidelity.[37] Nonetheless, the same structure suggests that the enzyme packs closely with the sugar residue of the bound nucleotide triphosphate through Y12,[37] which corresponds to F12 in Dbh, so that a 4′-alkyl substituent would not fit without perturbation of the local enzyme conformation (Figure 5). As a consequence, binding of a 4′-modified steric probe must be accompanied by a significant rearrangement of amino acid side chains.

Misincorporation of $T^{H}TP$ by KF^- led to a 10000-fold reduction in the single-turnover incorporation rate. These findings are in good agreement with earlier studies by Kuchta et al.[14] The nucleotide affinity decreased by a factor of 16. A comparison of the misincorporation of $T^{H}TP$ and $T^{Me}TP$ opposite a G template showed that the presence of the methyl group in the sugar moiety surprisingly caused an increase of about threefold in nucleotide binding, whereas the incorporation rate was reduced by about 14-fold. Again there are striking differences between HIV-1 RT and KF^-. Performing analogous experiments we found that the affinity of RT for $T^{Me}TP$ had dropped by 57-fold in relation to $T^{H}TP$, while the difference in incorporation was only twofold.[43]

Single-turnover misincorporation of $T^{H}TP$ by Dbh led to a 300-fold reduction in the incorporation rate. At the same time, nucleotide affinity merely dropped by a factor of two. Essentially, there was no drastic further reduction in rate when $T^{Me}TP$ was misincorporated. Notably, on misincorporation of $T^{H}TP$ by Dbh only about 30% of the primers were extended. This was even more pronounced when $T^{Me}TP$ was incorporated opposite to a G template. A possible explanation for this observation could be that a substantial percentage of substrates are bound in a nonproductive geometry. This could be due to missing Watson–Crick base pairing, which during correct incorporation might help in stabilizing the substrates in a catalytically competent orientation. As outlined above, there are few contacts of the enzyme to the bound nucleotide. Accordingly, without proper base pairing the substrate might end up misaligned within the active site. As described under "Results", no exact determination of the K_d value of $T^{Me}TP$ during misincorporation was possible. An increased affinity of $T^{Me}TP$ in relation to $T^{H}TP$, as has been observed for correct nucleotide insertion, would explain the additional drop in primer extension during misincorporation of this analogue through further stabilization of misaligned substrates. Such a scenario could also explain why we were unable to observe any incorporation of $T^{iPr}TP$ even opposite an A template. It is quite feasible that $T^{iPr}TP$ indeed binds to Dbh but is not incorporated because of severe substrate misalignment.

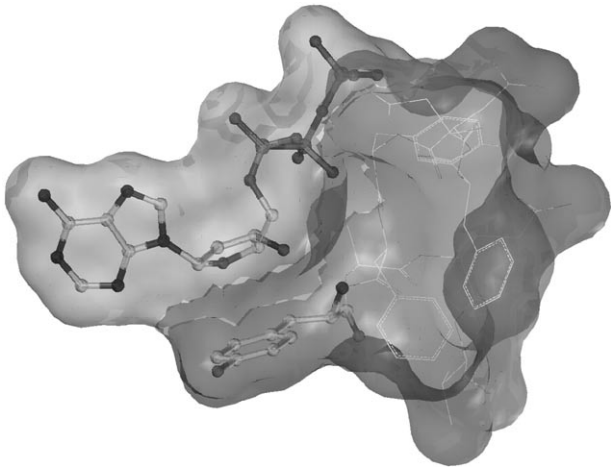


Figure 5. Bound dNTP in the ternary complex of Dpo4 DNA polymerase. The Connolly surfaces are shown in a close-up view highlighting the contact of the dNTP and Y12. The model is based on PDB code 2AGQ.[52]

During correct incorporation, both polymerases—KF^- as well as Dbh—tolerate all sugar-modified nucleotides up to the size of at least an ethyl group at the 4′-position. However, one should keep in mind that the two polymerases differ in their TTP incorporation efficiencies by a factor of about 7000 in favour of KF^- (see Table 1). As outlined above, $T^{iPr}TP$ binding to Dbh is likely but could not be experimentally proven. There is thus no major discrimination during the initial nucleotide binding step (formation of a low-affinity collision complex). Accordingly, nucleotide selection must occur during subsequent steps. Our own kinetic studies indicated that the rate-limiting step of nucleotide incorporation by Dbh is most probably not associated with a conformational change of the finger domain.[23] Therefore, the observed nucleotide incorporation rate most probably represents the chemical step. This finding

is in good agreement with a recent structural study of yeast Pol η in different complexes with a cisplatin–GG adduct.[48] The observed increase in binding affinity of the size-augmented probes during correct incorporation and a mere twofold drop in affinity during misincorporation can be explained in terms of a very flexible dNTP binding pocket in the bypass DNA polymerase. The fact that the alkyl groups at the 4′-position in the sugar moiety allow stronger interaction in the nucleotide binding pocket than the natural substrate leads to the conclusion that the observed reduction in the incorporation rate is probably a consequence of more unfavourable positioning for the nucleophilic attack of the primer 3′-hydroxyl group. These findings are consistent with a recent report of a Y-family DNA polymerase that employed nonpolar nucleobase surrogates with varied steric demand, in which small size preference was also found.[38] The analysis of misincorporation shows a similar picture. Here the nucleotide binding pocket accepts the noncomplementary nucleotide without a drastic change in incorporation rates.

For KF$^-$ the situation is less clear. There are several reports favouring a conformational change (closure of the finger domain to a tight ternary complex) as the rate-limiting step of correct nucleotide incorporation.[13,16,29,49–51] More recent studies have provided some evidence for the chemical step as being rate-limiting.[28] Interestingly, a recent study on Klentaq1 by Rothwell and Waksman implies the participation of the templating base in dNTP ground state selection: that is, an initial rearrangement of the templating base before dNTP binding and/or fingers subdomain closure.[46] Thio analogue experiments have suggested the chemical step to be rate-limiting during misincorporation.[14] Our data concerning correct incorporation of T^{H}TP, T^{Me}TP and T^{Et}TP are consistent with the concept of a conformational change being the major determinant of discrimination. However, we cannot currently entirely rule out the chemical step of nucleotide incorporation being rate-limiting for these T^{R}TP analogues, because the analogues are not well aligned for the nucleophilic attack such as we propose for Dbh. The high affinity of KF$^-$ for T^{iPr}TP clearly shows that this polymerase has a rather spacious nucleotide binding pocket able to accommodate even large modifications. Hence, the observed drastic drop in the incorporation rate might also be due to an unfavourable position of the substrate for nucleophilic attack. In contrast to Dbh, discrimination by KF$^-$ during misincorporation clearly arises through a reduced binding affinity for the nucleotide along with a reduced incorporation rate. Whether this reduced incorporation rate is affected by an induced-fit mechanism or misalignment remains to be elucidated.

In summary, despite the continuing lack of a rigorous understanding of the mechanism of DNA polymerase fidelity, our results support the notion that varied active site tightness is well suited to explain at least certain aspects of the varied selectivities and substrate spectra of the investigated DNA polymerases. Obviously, more work is needed in order to access whether these conclusions also hold true for other members of the investigated DNA polymerase families, as well as for other families.

Experimental Section

Proteins: Full-length Dbh was expressed and purified as described.[23] Recombinant KF$^-$ was expressed in *E. coli* strain M15. Cells were grown at 37 °C in LB medium (5 L), induced at an OD600 of 0.7 by addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) and harvested after 4 h. Cells were resuspended in buffer A [Tris/HCl (100 mM), pH 8.0, with NaCl (1 M)] and lysed by sonication with added phenylmethylsulfonyl fluoride (1 mM). After centrifugation the supernatant was loaded onto a Ni-NTA column (10 mL, Qiagen) and eluted by use of an imidazole gradient. KF$^-$-containing fractions were pooled and dialysed against buffer B [Tris/HCl (50 mM), pH 7.6, with NaCl (50 mM) and glycerol (10%)]. A further purification step included a S75 26/60 gel filtration column (GE Healthcare), yielding 99% pure protein. Enzyme concentration was routinely determined by use of an extinction coefficient at 280 nm of 55330 $M^{-1}cm^{-1}$ in buffer C [guanidinium hydrochloride (6 M) and sodium phosphate (20 mM), pH 6.5].

Buffers: All experiments were carried out at 25 °C in a buffer containing Tris/HCl (50 mM, pH 8.0) and $MgCl_2$ (10 mM). Annealing buffer consisted of Tris-HCl (20 mM, pH 7.5) and NaCl (50 mM).

Modified thymidine-5′-triphosphates: 4′-Modified thymidine-5′-triphosphates T^{R}TP were synthesized as described previously.[39]

Oligonucleotides: Oligodeoxynucleotides were purchased from IBA (Göttingen, Germany) and purified by denaturing polyacrylamide gel electrophoresis [acrylamide (15%), urea (7 M)], followed by elution from the gel by use of a Schleicher & Schuell Biotrap unit. The sequence of the 24/36-mer DNA/DNA primer/template (p/t) was 5′-GTGGTGCGAAATTTCTGACAGACA and 5′-GTGCGTCTG-TC**X**TGTCTGTCAGAAATTTCGCACCAC (X = A for correct insertion; X = G for misinsertion), respectively. Primer oligodeoxynucleotides were 5′-end-labelled with T4 polynucleotide kinase as described.[53] Primer and template oligodeoxynucleotides were annealed by heating equimolar amounts in annealing buffer at 90 °C, followed by cooling to room temperature over several hours in a heating block. The degree of completeness of the reaction was checked by determining whether 100% of the primer of the hybridized and radioactively labelled p/t could be extended by one nucleotide. The samples were analysed on denaturing gels.

Rapid kinetics of nucleotide incorporation: Rapid-quench experiments were carried out in a chemical quench-flow apparatus (RQF-3, KinTek Corp., University Park, PA, USA). Reactions were started by rapid mixing of the two reactants (15 μL each) and were then quenched with trifluoroacetic acid (TFA, 0.6%) at defined time intervals. All concentrations reported are final concentrations after mixing in the rapid quench apparatus. Products were analysed by denaturing gel electrophoresis [polyacrylamide (10%)/urea (7 M)] and quantified by scanning of the dried gel with use of a phosphor imager (Fuji FLA 5000). Data were evaluated by use of the program Grafit (Erithacus Software). For pre-steady-state kinetics, a preformed complex of p/t-polymerase [p/t (100 nM) with KF$^-$ (200 nM) and Dbh (1.5 μM), respectively] was rapidly mixed with an excess of dNTP (100 μM–4 mM) and stopped after various times in the millisecond–second range. The experimental data were fitted either to a single or to a double exponential equation [Eq (1)]:

$$[\text{product}] = A[1-\exp(-k_{pol}t)] \quad (1)$$

A is the amplitude of the burst, which reflects the concentration of active p/t-bound enzyme at $t = 0$. In the case of a biphasic burst of product formation the above equation was extended by a second exponential term. The effective pre-steady-state constants (k_{pol}) at

the given dNTP concentration are derived from the exponential rates.

Affinities of T^RTPs were determined from the dependence of the pre-steady-state burst rate on the T^RTP concentration. To measure the affinities of the T^RTPs the preformed p/t–polymerase complex was rapidly mixed with various concentrations of T^RTPs and quenched after $\tau_{1/2}$ of the maximal pre-steady-state rate. The corresponding rates were then calculated from the concentration of elongated primer by converting the exponential equation into:

$$k = -\ln \frac{1-([P+1]t/[P]0)}{t(s)} \quad (2)$$

$[P]_0$ corresponds to the concentration of polymerase–p/t complex available for incorporation at $t=0$ (burst amplitude), and t equals the reaction time ($\tau_{1/2}$ of the maximum pre-steady-state rate). The observed rates were plotted against the T^RTP concentration, and the dissociation constant (K_d) was calculated by fitting the data to a hyperbola.

Misincorporation kinetics: In the case of Dbh, the misincorporation experiments were performed manually. Reactions were started by mixing equal volumes (5 μL) of the two reactants and were then stopped with trifluoroacetic acid (0.6%) after defined time intervals. Products were analysed as described above. Dissociation constants were determined as described in the previous section with T^RTP concentrations in the 1 μM to 6 mM range.

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