Pre-steady-state Kinetic Characterization of the DinB Homologue DNA Polymerase of Sulfolobus solfataricus*

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Equilibrium as well as pre-steady-state measurements were performed to characterize the molecular basis of DNA binding and nucleotide incorporation by the thermostable archaeal DinB homologue (Dbh) DNA polymerase of Sulfolobus solfataricus. Equilibrium titrations show a DNA binding affinity of about 60 nM, which is ~10-fold lower compared with other DNA polymerases. Investigations of the binding kinetics applying stopped-flow and pressure jump techniques confirm this weak binding affinity. Furthermore, these measurements suggest that the DNA binding occurs in a single step, diffusion-controlled manner. Single-turnover, single dNTP incorporation studies reveal maximal pre-steady-state burst rates of 0.64, 2.5, 3.7, and 5.6 s⁻¹ for dTTP, dATP, dGTP, and dCTP (at 25 °C), which is 10–100-fold slower than the corresponding rates of classical DNA polymerases. Another unique feature of the Dbh is the very low nucleotide binding affinity (Kₜ ~ 600 μM), which again is 10–20-fold lower compared with classical DNA polymerases as well as other Y-family polymerases. Surprisingly, the rate-limiting step of nucleotide incorporation (correct and incorrect) is the chemical step (phosphoryl transfer) and not a conformational change of the enzyme. Thus, unlike replicative polymerases, an “induced fit” mechanism to select and incorporate nucleotides during DNA polymerization could not be detected for Dbh.

Polymersases involved in genome replication accomplish their task with high fidelity, ensuring genome stability. The low error rate in the range of 10⁻³ to 10⁻⁵ per base insertion can be augmented down to 10⁻⁷ by an intrinsic exonucleolytic proofreading strategy (1–3). However, these DNA replicases are not able to cope with certain DNA lesions (e.g. cyclobutane pyrimidine dimers resulting from UV damage), which represent a road block and would cause the entire replication machinery to stall. Thus, to ensure survival, all three kingdoms of life have evolved a complicated machinery, also known as translesion synthesis or lesion bypass, to overcome such blocks. In recent years, specialized DNA polymerases (Y-family polymerases) (4) have been discovered as the main components of this complex repair system (for a review, see Refs. 5 and 6). Although many aspects of the translesion synthesis phenomenon are not yet fully understood, there has been tremendous progress in the last few years. In vitro studies indicate that Y-family polymerases are particularly distensible enzymes accompanied by a remarkably high error rate in the range of 10⁻² to 10⁻³. This low fidelity is due to a loose polymerase active site and lack of fidelity-checking mechanisms (for a recent review, see Ref. 7). Thus, it appears that their ability to bypass lesions is at the cost of low fidelity. This in turn suggests that they have to be strictly regulated in order to prevent damage to the cell. There is growing evidence that these polymerases are recruited by the replication machinery through interactions with proliferating cell nuclear antigen in eukaryotes (8) or corresponding structures in other organisms (9) to bypass lesions during DNA synthesis (for a recent review, see Ref. 10).

The x-ray structures of three nonhuman Y-family DNA polymerase members, the N-terminal catalytic domain of yeast DNA polymerase η and Sulfolobus solfataricus (P1 and P2, respectively) DNA polymerases Dbh (DinB homologue) and Dpo4 (DNA polymerase IV), the latter crystallized in a ternary complex with DNA and an incoming nucleotide, reveal that these proteins have a high degree of structural homology (11–14). As seen for classical polymerases, these enzymes resemble a right-hand shape consisting of palm, fingers, and thumb domains. In addition, a unique but variable little finger domain is found. Deducing from the ternary Dpo4-DNA-nucleotide complex, the Y-family polymerases generally adopt a very open conformation, with the DNA substrate being solvent exposed near the active site and only a few contacts with the replicating base pair. As a consequence, the active site is rather spacious, enabling the enzymes to accommodate all kinds of distortions in the DNA template. However, each Y-family polymerase member differs in substrate specificity and as a result is specialized for a particular DNA lesion. It is assumed that the little finger domain plays a crucial role in defining catalytic properties and substrate specificity (15).

Here we used the Dbh DNA polymerase of S. solfataricus P1 as a model system to examine the molecular mechanism of the DNA polymerization reaction of Y-family DNA polymerases, which are universal to Bacteria, Archaea, and Eukarya. It should be noted that a recent reexamination concerning the origin of sequence of the dbh-containing fragment used for cloning of the recombinant enzyme (16) indicates a perfect match with the genomic sequence from S. acidocaldarius (15). Dbh therefore originates most likely from S. acidocaldarius and not S. solfataricus P1 as was originally thought. Nonetheless, in order to comply with recent literature, we will refer to it as S. solfataricus.

The aponzyme structure of Dbh suggests that the fingers domain is already in the “closed” active conformation instead of an “open” inactive conformation, as compared with the A and B family polymerases (12). This assumption is supported by homology modeling studies with the Dpo4 ternary complex (17).

In this study, we report on steady-state and pre-steady-state kinetic analyses, aiming to unravel mechanistic details of DNA binding and nucleotide incorporation by the Y-family Dbh polymerase. For the first time, we provide experimental evidence that nucleotide insertion and misinsertion by Dbh do not follow an induced “fit mechanism,” indicating a polymerase pathway different from that observed with classical DNA polymerases as well as with other Y-family polymerases.
Experimental Procedures

Protein—Full-length Dbh was expressed in the Escherichia coli strain BL21(DE3). Cells were grown at 37 °C in 5 liters of LB medium, induced at an A600 of 0.7 by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside, and harvested after 4 h. Cells were resuspended in buffer A (20 mM HEPES (NaOH), pH 7.5, 50 mM NaCl, 5 mM β-mercaptoethanol, and 0.5 mM EDTA) and lysed by sonication with 1 mM phenylmethylsulfonyl fluoride added. The lysate was then precipitated after heating the solution to 85 °C for 5 min to denature the host cell proteins. After centrifugation, the supernatant was loaded onto a HiTrap SP-Sepharose column (5 ml; Amersham Biosciences) and eluted via a NaCl gradient. The Dbh-containing fractions were pooled and dialyzed against buffer B (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, and 5 mM β-mercaptoethanol). A further purification step included a HiLoad 26/60 Superdex 75 gel filtration column (Amersham Biosciences), yielding 99% pure protein. Enzyme concentration was routinely determined using an extinction coefficient at 280 nm of 19,200 M⁻¹ cm⁻¹ in buffer C (6 mM guanidinium hydrochloride, 20 mM sodium phosphate, pH 6.5).

Protein Labeling—150 μl of a 80 μM protein solution (20 mM Tris-HCl, pH 7.6, and 50 mM NaCl) were mixed with 8 μl of a freshly prepared tetramethylrhodamine (Molecular Probes, Inc., Eugene, OR) solution (30 mM tetramethylrhodamine in dimethylsulfoxide). The reaction mixture was incubated for 1 h at 4 °C in the dark. Excess dye was removed by gel filtration applying a 30-ml Sephadex G-25 column (Amersham Biosciences) in buffer B (20 mM Tris-HCl, pH 7.6, and 1 mM NaCl). To determine the protein concentration and the labeling efficiency, the absorption at 280 and 541 nm was measured. The labeling efficiency was ~90%.

Buffers—All experiments were carried out at 25 °C, unless otherwise indicated, in a buffer containing 50 mM Tris-HCl, pH 8.0, and 10 mM MgCl₂. Annealing buffer consisted of 20 mM Tris-HCl, pH 7.5, and 50 mM NaCl.

Oligonucleotides—Oligodeoxynucleotides were purchased from a commercial supplier and purified by denaturing polyacrylamide gel electrophoresis (15% acrylamide, 7 M urea) followed by elution from the gel using the Schleicher & Schuell Biotrap unit.

The sequence of the 24/36-mer DNA/DNA p/t² was 5'-GTGGTGCGAAATTTC-TCCCT- GTGCAGAAAATCTCATGCAGAG-GCGGCAGAACAGGGA and the sequence of the fluorescently labeled 24/36-mer DNA/DNA p/t was 5'-TGTGGTGCGAAATTTC-TCCCT-GTGCGTCTGTCATATCTGTCAGAAAATTCTGCACCAC (where T* represents 5-C6-amino-2-thiodyrimidine linked to mansyl). Coupling of a mansyl fluorophor to an amino linker at positions 17 and 23 of the 18- and 24-mer primer, respectively, was performed as described previously (18). Primer oligodeoxynucleotides for the nucleotide incorporation studies were 5'-end-labeled with T4 polynucleotide kinase as described (19). 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 metal block. The completeness of the reaction was checked by determining whether 100% of the primer could be extended by one nucleotide. The samples were analyzed on 10% denaturing gels.

² The abbreviations used are: p/t, primer-template; Sp-α-S-dTTP, 2'-deoxythymidine-5'-O-(1-thiophosphosphate), Sp-isomer; α-S-dTTP, 2'-deoxythymidine-5'-O-(1-thiophosphosphate).

Nucleotides—dNTPs were purchased from Amersham Biosciences, and Sp-α-S-dTTPs was obtained from BioLog Life Science Institute (Bremen, Germany).

Fluorescence Titrations of Primer-Template Binding—Fluorescence titrations were performed using an SLM Smart 8100 spectrofluorimeter equipped with a PH-PC 9635 photomultiplier. Fluorescence of the mansyl group was excited at 365 nm, and the emission was monitored at 420 nm. The rhodamine chromophor was excited at 541 nm, and the emission was observed at 578 nm. Data were evaluated using the program Grafit (Erithacus Software). The relative fluorescence was plotted against the corresponding protein concentration, and the dissociation constant was calculated using a quadratic equation. All experiments were carried out at 25 °C.

To determine the affinity constant via competition experiments, a preformed complex of labeled p/t (68 nM) and Dbh (86 nM) was titrated with unlabeled p/t (where the asterisk indicates the covalently linked chromophor mansyl).

Rapid Kinetics of p/t Dbh Association—Concentration-dependent p/t Dbh association was analyzed using a homemade pressure jump apparatus as described elsewhere (20). A preformed p/t/Dbh complex (80 nM p/t and varying concentrations of Dbh; 0.4–1 μM) was exposed to 1000 pressure jumps (2–40 MPa). The kinetic time courses were observed via a cut-off filter (418 nm) after excitation of the mansyl fluorophor at 365 nm. The reassociation kinetic (pressure down) was analyzed, fitting the averaged experimental data to a single exponential equation.

Rapid Kinetics of p/t Dbh Dissociation—Stopped-flow experiments were carried out with a Hi-Tech SF-61 MX multimixing stopped-flow spectrofluorimeter at 25 °C. The mansyl chromophor of the labeled p/t (see above) was excited at 365 nm. Emission at 420 nm was detected via a 418-nm cut-off filter. Competition of the fluorescent labeled p/t was initiated by rapidly mixing equal volumes of preformed p/t/Dbh complex (80 nM p/t and 60 nM Dbh) with an excess of unlabeled p/t (900 nM). All concentrations reported are final concentrations after mixing in the stopped-flow cell. Data were evaluated using the program Grafit.

Rapid Kinetics of Nucleotide Incorporation—Rapid quench experiments were carried out in a chemical quench flow apparatus (RQF-3, KinTek Corp., University Park, PA). Reactions were started by rapidly mixing the two reactants (15 μl each) and then quenched with 0.6% trfluoroacetic acid (Ft) for 15 s. Additional experiments were performed using a phosphor imager (Fuji FLA 5000). Data were evaluated using the program Grafit (Erithacus Software).

For pre-steady-state kinetics, a preformed complex of p/t/Dbh (100 nM p/t and 1.5 μM Dbh) was rapidly mixed with an excess of dTTP (100 μM to 6 μM) and stopped after various times in the millisecond to second range. Data were fitted to a single exponential burst equation. The effective pre-steady-state constants (kseg) at the given dNTP concentration are derived from the exponential rate.

Affinity of dTTP with the binary Dbh-p/t complex was determined by the dependence of the pre-steady-state burst rate on the dTTP concentration. To measure the affinity of the dTTP, the preformed p/t/Dbh (100 nM and 1.5 μM) complex was rapidly mixed with various concentrations of dTTP and quenched after t½ 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[1 − ([P] − [P]t)/[P]t] (at (P) correspond to the concentration of Dbh-p/t complex available for incorporation at t = 0 (burst amplitude),
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FIGURE 1. Equilibrium titrations of DNA substrate with Dbh. A, a 24/36 DNA/DNA p/t (80 nM) was titrated with Dbh while monitoring the mansyl fluorescence of the labeled primer (excited at 365 nm and observed at 420 nm). B, tetramethylrhodamine-labeled Dbh (37.5 nM) was titrated with p/t (excitation at 541 nm; emission at 579 nm). The curves show the best fit of the data to a quadratic equation. The analysis of the data yielded a dissociation constant ($K_d$) of 61.6 ± 2.0 nM (A) and 58.9 ± 12.9 nM (B), respectively.

and $t$ equals the reaction time ($t_{1/2}$) of the maximal pre-steady-state rate. The observed rates were plotted against the dTTP concentration, and the dissociation constant ($K_d$) was calculated by fitting the data to a hyperbola.

Misincorporation Kinetics—Misincorporation experiments were performed manually. Reactions were started by mixing equal volumes (5 μl) of the two reactants and then quenched with 0.6% trifluoroacetic acid after defined time intervals. Products were analyzed as described above. The dissociation constant was determined as described under “Rapid Kinetics of Nucleotide Incorporation,” using dTTP concentrations in the range of 1 μM to 6 mM.

RESULTS

Interaction of Dbh with Primer-Template under Steady-state Conditions—To investigate the binding affinity of Dbh to DNA, equilibrium titrations with a 24/36 DNA/DNA p/t were performed, using a mansyl chromophor attached to the primer as probe (p*). Fig. 1A shows a typical titration curve of the mansyl-labeled p/t molecule with Dbh. Titration of a fixed p*/t concentration (80 nM) with increasing amounts of Dbh resulted in an increase of fluorescence of about 70%. By fitting the experimental data to a quadratic equation, an equilibrium constant ($K_d$) of 61.6 ± 2.0 nM for this interaction could be obtained. Using a 18/36 DNA/DNA p/t comprising an entirely different sequence (see under "Experimental Procedures") a $K_d$ of 55.4 ± 6.0 nM was obtained (data not shown). Interestingly, the affinity of Dbh to DNA is surpris-ingly low compared with other DNA polymerases with typical $K_d$ values in the low nanomolar range. To exclude the possibility that the observed signal increase was caused by nonspecific interactions between the fluorescence label and the Dbh, we performed fluorescence titrations of tetramethylrhodamine-labeled Dbh with unlabeled p/t.

Fig. 1B shows a titration curve of 80 nM Dbh with increasing amounts of p/t. The DNA binding resulted in a quenching of the fluorescence signal of ~25%. The curve shows the best fit of the experimental data to a quadratic equation, yielding a binding affinity ($K_d$) of 58.9 ± 12.9 nM.

A further method to confirm the observed $K_d$ of the labeled DNA to Dbh is to determine the binding affinity of an unlabeled p/t indirectly via competition experiments (19). Thus, a preformed complex of p*/t/Dbh (68 nM p*/t and 86 nM Dbh) was titrated with unlabeled p/t (Fig. 2). Data were fitted to a multi-equilibrium model, yielding a binding affinity of $52.7 ± 7.1$ nM. This indirectly derived dissociation constant again corresponds very well with the data obtained from the direct titrations.

Time-resolved Characterization of p/t Binding—To characterize the DNA binding reaction in more detail, stopped-flow experiments were performed. First, we examined whether the p/t Dbh association occurs in a concentration-dependent manner. To address this question, equal volumes of p*/t (80 nM) and Dbh (400–1000 nM) solutions were rapidly mixed, and the time course of the fluorescence change was monitored. Surprisingly, the binding kinetics was so fast that merely the last 20% of the reaction could be resolved. Accordingly, a proper evaluation of the experimental data was not possible by applying this technique. Thus, a pressure jump apparatus was used to resolve the fast association kinetic of p/t and Dbh. The time resolution of such a device is about 10–20 times higher as compared with a stopped-flow apparatus. Preincubated complexes of p*/t/Dbh (80 nM p*/t and 400–1000 nM Dbh) were applied to 1000 pressure jumps. A representative time course of the relaxation kinetics of a p*/t/Dbh complex after pressure jumps down (900 nM Dbh and 80 nM p*/t) is shown in Fig. 3A. The association kinetics suggests a one-step p/t binding mechanism. The best fit to a single exponential equation resulted in a pseudo-first-order rate constant ($k_{on}$) of $1210 ± 35$ s⁻¹. The secondary plot of the dependence of $k_{on}$ on the Dbh concentration shows that the observed rates are concentration-dependent.

The data were fitted to a linear regression, yielding a slope of $1.35 ± 0.12$ nM⁻¹ s⁻¹, which corresponds to the association rate ($k_{on}$). This association rate suggests that p/t binding of Dbh is a very fast process and occurs in a diffusion-controlled manner.
the correct incorporation of dATP, dGTP, and dCTP into a 24/36 DNA/ 
pol of 2.5
H11002
1, 3.7 ± 0.6, and 5.6 ± 0.6 s⁻¹, respectively was obtained (data not shown).

Surprisingly, performing an equivalent incorporation experiment as 
described above under multiple-turnover conditions (excess of p/t over 
ww H11002
1, 0.03 and 0.07
H11006
1, 0.003 s⁻¹ for dTTP and Sp-α-S-dTTP, respectively, B, a preformed p/t-Dbh complex (100 
w H11006
1, 0.64 ± 0.03 s⁻¹) was rapidly mixed with increasing amounts of dTTP (C, 100– 
4000 μM) or Sp-α-S-dTTP (D, 100–6000 μM). The calculated rates were plotted against 
the respective dNTP concentrations and fitted to a hyperbola yielding a
kH11006
1 of 591 ± 55 μM in the case of dTTP and 334.4 ± 16.2 μM in the case of Sp-α-S-dTTP. The observed 
rates given on the left y axis correspond to dTTP, and the ones on the right correspond to 
Sp-α-S-dTTP.

(kH11002 out kH11006 out) would suggest that a burst is not observed. A likely expla-
nation for this finding is a considerably reduced off-rate in the presence of 
high concentrations of nucleotide representing the rate-limiting step 
of multiple-turnover nucleotide incorporation. To confirm that the 
observed slow phase indeed represents the steady-state phase, we per-
formed an equivalent incorporation experiment with the exception that 
the preformed enzyme-substrate complex was rapidly mixed with 
nucleotide in the presence of a competitor substrate (5 μM unlabeled 

FIGURE 3. Pre-steady-state kinetic analysis of Dbh and p*/t association. A, representative 
time course of the association kinetic of 900 nM Dbh and 80 nM p*/t observed after 
a pressure jump (from 40 to 2 MPa). The curve shows the best fit to a single exponential 
equation, resulting in a dissociation rate (kH11006 out) of 62.7 ± 2.1 s⁻¹. B, dependence 
of the fitted kH11006 out on Dbh concentration. Data were analyzed by linear regres-
sion, yielding an association constant (kH11002 in) of 1.35 ± 0.12 nm⁻¹ s⁻¹.

FIGURE 4. Analysis of dissociation of a fluorescently labeled p*/t from Dbh. A pre-
formed complex of Dbh (60 nM) and p*/t (80 nM) was rapidly mixed with an excess of 
unlabeled p/t (900 nM). The solid line shows the best fit of the data to a single exponential 
equation, resulting in a dissociation rate (kH11006 out) of 0.64 ± 0.03 s⁻¹.

FIGURE 5. Single-turnover, single dTTP, or Sp-α-S-dTTP incorporation into 24/36 
DNA/DNA p/t by Dbh and dependence of the pre-steady-state burst rate on dTTP or 
Sp-α-S-dTTP incorporation. A, the curves show the best fit of the experimental data to 
a single exponential equation. A preformed complex of 100 nM p/t and 1.5 μM Dbh was 
rapidly mixed with either 3 mM dTTP (□) or 3 mM Sp-α-S-dTTP (○). Analysis of the data by 
fitting to a single exponential equation yielded a burst rate of 0.64 ± 0.03 s⁻¹ for dTTP and Sp-α-S-dTTP, respectively. B, a preformed p/t-Dbh complex (100 

 μM p/t and 1.5 μM Dbh) was rapidly mixed with increasing amounts of dTTP (C, 100– 
4000 μM) or Sp-α-S-dTTP (D, 100–6000 μM). The calculated rates were plotted against 
the respective dNTP concentrations and fitted to a hyperbola yielding a
kH11006
1 of 591 ± 55 μM in the case of dTTP and 334.4 ± 16.2 μM in the case of Sp-α-S-dTTP. The observed 
rates given on the left y axis correspond to dTTP, and the ones on the right correspond to 
Sp-α-S-dTTP.
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FIGURE 6. Single-turnover kinetics of misincorporation of dTTP opposite a template G into 24/36 DNA/DNA p/t by Dbh (A) and dependence of the pre-steady-state burst rate on dTTP misincorporation (B). Preformed complexes of 100 nM p/t and 1.5 μM Dbh were rapidly mixed with 4 mM dTTP and stopped after definite time intervals (A). The experimental data were fitted to a single exponential equation, giving a misincorporation rate of 0.0017 ± 0.0001 s⁻¹. Increasing amounts of dTTP (400–6000 μM) were rapidly mixed with the preformed complex described above and quenched after ς, of the maximal pre-steady-state rate (B). Fit of the data to a hyperbola yielded a Kd of 1299 ± 100 μM.

24/36 DNA/DNA p/t. The addition of this trap leads to a complete disappearance of the slow phase (data not shown).

dTTP Binding Affinity for Correct Nucleotide Insertion—To ensure that the observed single-turnover rate of incorporation was not limited by binding parameters, we carefully examined the binding affinities of the incoming dTTP. The affinity was calculated by plotting the observed rates at various concentrations of dTTP and fitting the data to a hyperbola. The curve in Fig. 5B shows the best fit to a hyperbolic equation, resulting in a dissociation constant (Kd) of 591 ± 55 μM.

Single-turnover Nucleotide Misincorporation onto Template G—Next, we examined the misincorporation rate of dTTP opposite a template G to gain insight into the mechanistic details of nucleotide misincorporation of Dbh. Because of the slow misincorporation rate, it was not necessary to use a chemical quench flow apparatus. The amount of 25-mer product was plotted as a function of time and fitted to a single exponential equation. Fig. 6A shows the best fit to a single exponential equation, yielding a misincorporation rate of 0.0017 ± 0.0001 s⁻¹.

dTTP Binding Affinity for Incorrect Nucleotide Insertion—As already outlined above, it is essential to ensure that the incorporation studies are carried out at saturating nucleotide concentrations. We therefore also examined the binding affinities of the incoming nucleotide for the incorrect insertion opposite template G. The rate dependence on the concentration of dTTP was determined by plotting the calculated rates at various concentrations of dTTP and fitting the data to a hyperbola (Fig. 6B). The best fit yielded a dissociation constant (Kd) of 1299 ± 100 μM.

Elemental Effect for Correct and Incorrect Nucleotide Insertion—The next step was to investigate the underlying mechanism of the observed incorporation rates. The rates may reflect a rate-limiting conformational change prior to the chemical step, as it had been observed for other polymerases (e.g. HIV-1 RT, Klenow fragment, T7 DNA polymerase) (21–23). On the other hand, the rates could be due to a rate-limiting chemical step. To distinguish between these two possible scenarios, we examined the effect of a sulfur atom substitution for oxygen at the α-phosphate of dTTP on the incorporation rate of correct and incorrect insertion.

Analogous to the experiments described above, we determined the correct and incorrect nucleotide incorporation kinetics of α-S-dTTP into a 24/36 DNA/DNA p/t substrate under single-turnover conditions. Besides, we measured the binding affinity for α-S-dTTP in order to ensure that the incorporation studies are carried out at saturating nucleotide concentrations. The rate dependence on the concentration of α-S-dTTP was determined by plotting the calculated rates at various concentrations of α-S-dTTP and fitting the data to a hyperbola (Fig. 5B). The best fit yielded a dissociation constant (Kd) of 334.4 ± 16.2 μM. Fig. 5A shows the time courses of the product formation for the correct incorporation of dTTP and α-S-dTTP by Dbh. The best fit of the experimental data to a single exponential equation yielded an incorporation rate of 0.07 ± 0.003 s⁻¹ for α-S-dTTP. Compared with the incorporation rate of dTTP, the single nucleotide incorporation rate of α-S-dTTP was about 9-fold slower. Thus, the correct nucleotide incorporation shows an elemental effect, suggesting that the chemical step is rate-limiting.

For the incorrect α-S-dTTP incorporation, the elemental effect was so high that we could not observe any product formation over a long period of time (>3 h). Since product formation during incorrect insertion of α-S-dTTP was below the detection limit, we were not able to determine a true value for this large elemental effect. To verify that α-S-dTTP does bind to the polymerase under these conditions, we checked if the modified nucleotide inhibits dTTP insertion opposite G. At a concentration of 2 mM dTTP in the presence of 2 mM α-S-dTTP, we observed a reduction of product formation by about 50% (data not shown).

DISCUSSION

In this study, we have examined fundamental parameters of the DNA binding and nucleotide incorporation characteristics of the archaeal Y-family DinB homologue (Dbh) DNA polymerase of S. solfataricus.

Performing equilibrium fluorescence titrations and competition experiments, we determined a DNA binding affinity of a 18/36 and 24/36 p/t to Dbh in the range of 50–60 nM. This Kd value is surprisingly low compared with 2 nM in case of human immunodeficiency virus type 1 reverse transcriptase (24) and 0.3 nM in case of the Klenow fragment (25). This weaker binding affinity is consistent with the small number of contacts that have been predicted by modeling studies between Dbh and p/t based on the x-ray structure of the apoenzyme (12). A recent study reports a Kd of 10.6 nM for DNA binding by the Dpo4, another archaean Y-family DNA polymerase (26). The x-ray structure of this polymerase in complex with DNA substrate and incoming nucleotide shows only a few van der Waals contacts with the replicating base pairs and no contact with bases already passed through the active site (14). The DNA binding affinity of yeast pol β, a third Y-family DNA polymerase, has been reported with 11 nM (27). Here, as for Dbh, no crystal structure of a binary/ternary complex is currently available. Thus, the
$K_d$ values for DNA binding of Dpo4 and yeast pol η are ~6-fold lower compared with Dbh, suggesting that there might be more stabilizing interactions. A likely candidate for these additional interactions is the so-called “little finger” domain of Y-family polymerases (15).

Analyzing the association kinetics with the pressure jump technique, we found that association of Dbh and p/t occurs in a diffusion-controlled, one-step binding reaction with an astoundingly fast association rate of $1.35 \pm 0.12 \text{ s}^{-1}$. The dissociation rate was determined by competition experiments using a stopped-flow apparatus and yielded a $k_{off}$ of $62.7 \pm 0.12 \text{ s}^{-1}$. When calculating the affinity constant ($K_d$) of the DNA binding using the ratio of the dissociation to the association rate constant, we could observe a striking agreement with the data obtained from the equilibrium titrations. The observed fast dissociation rate fits well with the observation that Dbh is a rather distributive polymerase (12). This of course is not further surprising, since it is assumed that the natural function of this enzyme is to bypass certain destructive DNA lesions during DNA synthesis. As soon as this task is fulfilled, the replication machinery takes over and continues DNA synthesis with high processivity and fidelity. In that respect, a fast off-rate might protect the cell against dangerous mutations introduced by error-prone Y-family polymerases. On the other hand, in the presence of artificially high concentrations of nucleotide (>1 mM), the situation is different. In this case, the dissociation rate is reduced considerably (see below).

In a second set of experiments, nucleotide binding and incorporation were analyzed. Besides the “correct” incorporation generating a canonical base pair, misincorporation was analyzed. Single nucleotide incorporation studies under multiple-turnover conditions (100 nM enzyme and 1.5 μM p/t) occurred in a monophasic burst of product formation followed by a slower linear phase (data not shown). The slower phase with a rate of about 0.2 s$^{-1}$ represents the steady-state phase corresponding to the dissociation of the enzyme-substrate complex. This was confirmed by performing a trap experiment. Here the preformed enzyme-substrate complex was rapidly mixed with nucleotide in the presence of a competitor substrate. Therefore, nucleotide incorporation due to enzyme that has to dissociate and rebind is abrogated. In the presence of the trap, the slow phase is no longer observable (data not shown). The slower phase represented the rate-limiting step of nucleotide incorporation with a rate of about 0.02 s$^{-1}$, which is 0.1, 0.6, and 5.6-fold slower compared with classical DNA polymerases as well as viral polymerases indicated that several important conformational transitions occur during the polymerization reaction (for a review, see Ref. 33). First, p/t binds to the polymerase in a two-step process. Subsequent nucleotide binding to this complex also occurs in two steps before the nucleotide is incorporated during the catalytic step (phosphoryl transfer). After initial binding of the nucleotide to the polymerase-p/t complex, an isomerization of the fingers domain is induced, leading to a tight ternary complex. This isomerization represents the rate-limiting step of nucleotide incorporation, referred to now as an “induced fit” mechanism. Despite this, the available crystal structures of three low fidelity Y-family DNA polymerases allowed several authors to speculate that a conformational rearrangement of the fingers subdomain after initial nucleotide binding does not take place within this family (for a review, see Ref. 17). This is primarily based on a comparison of the apo-Dbh structure with the ternary Dpo4-DNA-nucleotide complex, suggesting that the fingers and palm domain maintain a closed (catalytically competent) conformation even without substrates (12–14). Thus, it has been proposed that the absence of an induced fit mechanism could be a unique feature of error-prone DNA polymerases to allow for translesion synthesis.

To determine which step in the Dbh polymerase reaction pathway is rate-limiting, incorporation studies with Sp-α-S-dTTP were performed. The sulfur atom possesses a lower electronegativity relative to oxygen, so that the nucleophilic attack of the 3′OH group of the primer is hampered. In the case of a conformationally controlled reaction, there would be no effect or only a slight one. A significant decrease in the incorporation rate would suggest a chemically controlled reaction step because of the elemental effect of the sulfur atom. In the past, rather large elemental effects of about 50–100-fold were interpreted as indication for a chemically controlled reaction (23, 34, 35). Other studies, however, indicate that an elemental effect of 4–11-fold is sufficient to suggest the chemical reaction to be rate-limiting (36). However, the assessment of mechanistic aspects of enzymatic reactions based on such experiments remains problematic (for a review, see Ref. 33).

Performing Sp-α-S-dTTP incorporation experiments, an elemental effect of 9.1 was observed incorporating the correct nucleotide. During misinsertion of α-S-dTTP opposite template G, the elemental effect was
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too large to be determined accurately. There was virtually no incorporation observable for an incubation time of 3 h, suggesting an elemental effect of 100-fold or more. Despite the above described intricacy, we interpret our data as pointing toward a chemical controlled reaction. On the other hand, a kinetic analyses of Dbh by Potapova et al. (29) yielded an elemental effect of about 4-fold for both the correct and incorrect incorporation. The authors speculate either the chemical step is not rate-limiting even with an incorrect nucleotide, or the chemical step is not associated with as large an elemental effect as in the classical polymerases. Furthermore, studies on yeast DNA polymerase η provide convincing evidence for a two-step mechanism of nucleotide incorporation (27). This was also reported for Dpo4 when incorporating the correct nucleotide (26). Thus, there is obviously some discrepancy concerning this issue among the Y-family DNA polymerases characterized so far that leaves room for speculation. Whether the different Y-family polymerase members indeed may have developed different strategies for selecting the nucleotide substrate remains to be seen.

In summary, analyzing mechanistic properties of the Dbh DNA polymerase, we present kinetic evidence in support of the current view for selecting the nucleotide substrate remains to be seen.

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