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Cooperation between Catalytic and DNA Binding Domains Enhances Thermostability and Supports DNA Synthesis at Higher Temperatures by Thermostable DNA Polymerases

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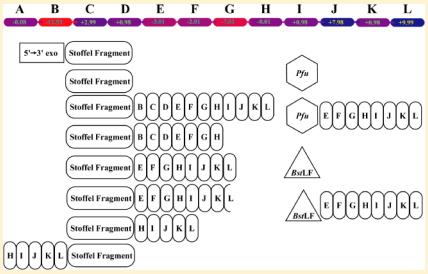
Supporting Information

ABSTRACT: We have previously introduced a general kinetic approach for comparative study of processivity, thermostability, and resistance to inhibitors of DNA polymerases [Pavlov, A. R., et al. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 13510−13515]. The proposed method was successfully applied to characterize hybrid DNA polymerases created by fusing catalytic DNA polymerase domains with various sequence-nonspecific DNA binding domains. Here we use the developed kinetic analysis to assess basic parameters of DNA elongation by DNA polymerases and to further study the interdomain interactions in both previously constructed and new chimeric DNA polymerases. We show that connecting helix−hairpin−helix (HhH) domains to catalytic polymerase domains can increase thermostability, not only of DNA polymerases from extremely thermophilic species but also of the enzyme from a faculatative thermophilic bacterium *Bacillus stearothermophilus*. We also demonstrate that addition of Topo V HhH domains extends efficient DNA synthesis by chimerical polymerases up to 105 °C by maintaining processivity of DNA synthesis at high temperatures. We found that reversible high-temperature structural transitions in DNA polymerases decrease the rates of binding of these enzymes to the templates. Furthermore, activation energies and pre-exponential factors of the Arrhenius equation suggest that the mechanism of electrostatic enhancement of diffusion-controlled association plays a minor role in binding of templates to DNA polymerases.



Thermostable DNA polymerases play a crucial role in current methods of DNA amplification and sequencing.[1] Major improvements in these methods have been made in the past 20 years since the introduction of *Taq* DNA polymerase, largely as a result of a significant number of new DNA polymerases available to scientists, and a marked improvement in the understanding of how DNA polymerases function in DNA sequencing and polymerase chain reactions. Many of the currently available thermostable polymerases were discovered through screening thermophilic organisms and phages for thermostable enzymes. The current trend in making new and improved DNA polymerases, however, is to apply rational redesign strategies based on introducing single mutations and/or domain swapping or tagging to construct new variants of the existing thermostable DNA polymerases.[1−3]

Our research has for several years been directed toward creating new DNA polymerases by "domain swapping" and "domain tagging" techniques, which provide large modifications of protein structure that cannot be achieved by point mutations or by small sequence insertions or deletions.[4−6] New DNA polymerases were obtained by the flexible attachment of helix−hairpin−helix (HhH) DNA binding domains of topoisomerase V (Topo V) of *Methanopyrus kandleri*[7−9] to catalytic domains of DNA polymerases.[5] The potential of this domain fusion approach was demonstrated using the *Taq* and *Pfu* DNA polymerases, and recently using the ϕ29 DNA polymerase,[10] which belong to different structural families and work in different temperature ranges. This method produced polymerases that retain high processivity at high concentrations of salts and other inhibitors of DNA synthesis, such as phenol, blood, and DNA intercalating dyes.[5] In addition, it was demonstrated[6] that attaching additional domains has the potential to greatly increase the thermostability of chimeric DNA polymerases.

We have developed a novel kinetic assay, which is based on time-dependent elongation of fluorescent primer−template junction (PTJ) substrates by DNA polymerases.[6] Quantitation of the products of polymerization on a DNA sequencer together with a corresponding procedure of computating initial rates of DNA polymerization allowed for a standard kinetic analysis of the elongation.

For comparative analysis of hybrid polymerases and their natural counterparts, we introduced a quantitative approach for assessment of the general effects of salt-containing buffers on the processivity of DNA polymerases. This approach is a further development of a model for processive DNA synthesis in replication described previously by von Hippel and co-workers.[11,12] The processivity equivalence parameter, P_e, which was introduced in our previous work,[6] can be calculated independently for all studied DNA polymerases and thus can serve a quantitative measure of processivity for these enzymes.

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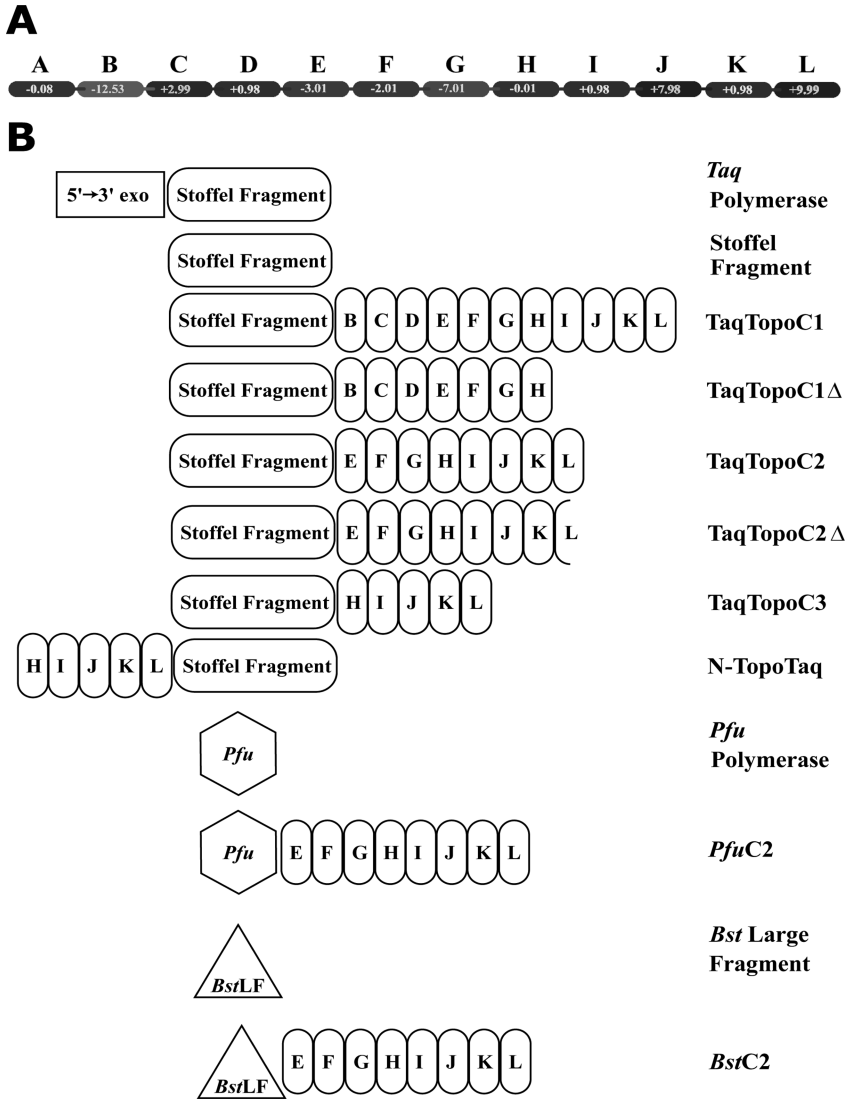

Figure 1. Schematic representation of chimeric DNA polymerases. (A) HhH domains of Topo V with calculated partial charges at pH 7.0. (B) Chimeric DNA polymerases, their catalytic domains, and original enzymes used in this study. HhH repeats are designated as in refs 6 and 7.

In this paper, we present a study and analysis of the effects of interdomain interactions in previously constructed hybrid enzymes and in new chimeric DNA polymerases, including the polymerase constructed by fusion of the *Bacillus stearothermophilus* (*Bst*) DNA polymerase and Topo V HhH domains.

MATERIALS AND METHODS

Taq DNA polymerase was purchased from Roche Applied Science (Indianapolis, IN); the Stoffel fragment of *Taq* DNA polymerase was obtained from Applied BioSystems (Foster City, CA), and *Pfu* DNA polymerase was from Stratagene Cloning Systems (La Jolla, CA). The recombinant large fragment of *Bst* DNA polymerase (IsoTherm DNA polymerase) was purchased from Epicenter Technologies (Madison, WI). Fluorescent M13 single-stranded DNA and ALF M13 Universal Primers were purchased from Amersham Biosciences (Piscataway, NJ). All other primers were synthesized at Fidelity Systems, Inc.

The gene of DNA polymerase family B from *M. kandleri* AV19 (*Mka* polB) was obtained through the polymerase chain reaction (PCR) amplification of genomic DNA.[13] The PCR product was digested with *Nco*I and *Eco*RI and cloned into expression vector pET21d. The integrity of the constructed vectors was verified by DNA sequencing.

Chimeric DNA Polymerases. Construction, expression, and isolation of chimeric DNA polymerases TopoTaq, TaqTopoC1, TaqTopoC2, TaqTopoC3, and *Pfu*C2 were described in our previous paper.[6] DNA polymerase TaqTopoC1Δ that differs from TaqTopoC1 in lacking HhH repeats H−L, and TaqTopoC2Δ obtained from TaqTopoC2 by cutting off 16 C-terminal positively charged amino acid residues, were prepared as follows: DNA fragments containing truncated HhH C1 and C2 tails were produced by PCR amplification using expression vectors TaqTopoC1-pET21d and TaqTopoC2-pET21d,[6] respectively, as templates, with the T7 promotor primer and primers that insert a stop codon with a *Hin*dIII site into defined regions of DNA. The PCR products were then cut with *Hin*dIII, and the DNA sequences coding the truncated tails were inserted into expression vector TaqTopoC2-pET21d, which was cleaved using *Hin*dIII. The orientation and sequence of the tails were verified by DNA sequencing.

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Genomic DNA of *B. stearothermophilus* Donc (ATCC12980D-5) was obtained from ATCC (Manassas, VA). Because sequences of DNA polymerase for various strains of *B. stearothermophilus* exhibit considerable differences, we designed several inward-directed PCR primers to the ends of the polymerase gene, using data from GenBank. The longest PCR product was selected and sequenced. To determine the flanking sequences for the *Bst* DNA polymerase gene, two outward Fimers complementary to the ends of the PCR product were synthesized and used for direct genomic sequencing.[14] Then, the region of DNA encoding the *Bst* polymerase domain was amplified by PCR with the following primers: CGAAAAGCCGCTCGCCGCCATGGATTTTGC (primer 1) and GGCAGGAAGCTTAATTTGGCGTCGTACCACG (primer 2).

Primer 1 creates an *Nco*I restriction site followed by an initiation codon for the large fragment (LF) of the *Bst* DNA polymerase, and primer 2 incorporates a *Hin*dIII site downstream from the termination codon of the *Bst* DNA polymerase gene. The PCR product was verified by DNA sequencing. As the amplified region had internal *Nco*I and *Hin*dIII sites, the following cloning strategy was applied: (i) Expression vector TaqTopoC2-pET21d was digested with *Nco*I and *Hin*dIII, and two DNA fragments were purified: a *Hin*dIII−*Hin*dIII fragment encoding the C2 tail of TopoTaqC2 and an *Nco*I−*Hin*dIII fragment of vector pET21d. (ii) The PCR-amplified DNA encoding LF *Bst* DNA polymerase was cut with *Nco*I, and two large pieces were separated. The fragment encoding the C-terminal fragment of the LF *Bst* polymerase gene was then treated with *Hin*dIII and inserted between the *Nco*I and *Hin*dIII sites of the *Nco*I−*Hin*dIII fragment of pET21d. (iii) The resulting construct was then cut with *Hin*dIII; the C2 domain-coding region was inserted between two *Hin*dIII sites, and its orientation was verified by sequencing. (iv) The plasmid was then cut with *Nco*I, and the *Nco*I−*Nco*I fragment of the PCR product encoding the N-terminal part of the LF *Bst* polymerase gene was inserted between two *Nco*I sites. The final construct, expression vector *Bst*C2-pET21d, was verified by sequencing.

TaqTopoC1Δ, TaqTopoC2Δ, *Mka* PolB, and *Bst*C2 were expressed in *Escherichia coli* strain BL21 pLysS and purified to apparent homogeneity [>95% pure, according to sodium dodecyl sulfate−polyacrylamid gel electrophoresis (SDS−PAGE)].[15] The cell lysates, prepared as described previously,[6] were heated at 75 °C for 20 min (for TaqTopoC1Δ, TaqTopoC2Δ, and *Mka*PolB) or at 60 °C for 20 min (for *Bst*C2), cleared by ultracentrifugation, and then applied to a heparin High Trap column (Amersham Pharmacia Biotech) equilibrated with 0.25 M NaCl in 50 mM Tris-HCl buffer (pH 7.5) at 25 °C. The proteins were eluted with a linear gradient from 0.25 to 1 M NaCl. Fractions containing the *Bst*C2 protein were combined, diluted to 0.15 M NaCl, and applied to a Q-Sepharose High Trap 1 mL column equilibrated with a buffer containing 50 mM Tris-HCl (pH 8.5), 0.15 M NaCl, and 2 mM β-mercaptoethanol. The polymerases were eluted using a linear gradient from 0.15 to 1 M NaCl and concentrated with Centricon YM-30 (Millipore). Figure 1 presents a sketch of the catalytic and DNA binding domains for chimeric and natural polymerases used in this study.

Protein Concentrations. The concentrations of the expressed and purified DNA polymerases were determined by the modified Lowry protein assay (Pierce) and compared to the quantitation of the protein bands resolved by SDS−PAGE,[15] using different concentrations of bovine serum albumin (Sigma) as a reference in both methods. The protein concentration of commercial DNA polymerases was determined by PAGE followed by quantitation in the gels.

Melting Temperatures of Duplexes. The melting temperatures of duplexes were measured on a Varian 300 spectrophotometer with the heated block in primer extension reaction mixtures, using temperature gradients of 0.2 °C/min and continuous recording of the optical density at 254 nm, and calculated as medians of the inflection points on the heating and cooling curves. Each melting experiment was conducted in triplicate.

Primer Extension Assays. The DNA polymerase activity, the initial rates of extension (v_1), and the processivity parameters for the reactions (P_e) were measured and calculated as described previously.[6] Briefly, DNA polymerase reaction mixtures (15−20 μL) contained dATP, dTTP, dCTP, and dGTP (1 mM each), 4.5 mM $MgCl_2$, detergents Tween 20 and Nonidet P-40 (0.2% each), fixed concentrations of PTJ duplexes, other additions, as indicated, and appropriate amounts of DNA polymerases in 30 mM Tris-HCl buffer (pH 8.0) at 25 °C. The background reaction mixtures contained all the components except the polymerases. Primer extensions were conducted for a preset time in a PTC-150 minicycler (MJ Research). For each reaction, 5 μL samples were removed after incubation for 2, 4, and 8 min and chilled to 4 °C, followed by immediate addition of 20 μL of 20 mM EDTA. The samples were desalted by centrifugation through Sephadex G-50 spin columns, diluted, and analyzed on an ABI Prism 377 DNA sequencer (Applied Biosystems). The products of elongation were quantitated after integration of fluorescent profiles of the electrophoresis, and the initial rates of extension were calculated from the progressive accumulation of the products, as described by Boeker.[16] For calculation of the processivity parameters, after determination of the amount of extended products, the initial rates for the appearance of each elongated product were calculated.

The substrate for the standard assay was prepared by annealing a 5′-end, labeled with a fluorescein 20-nucleotide primer, with a 30-nucleotide template:

```
Fl*-GTAATACGACTCACTATAGGG  -> extension
     ||||||||||||||||||||
     CATTATGCTGAGTGATATCCCaaaaaccccc          (PTJ1)
```

The standard assay was conducted at 70 °C, and the reactions were started by the addition of DNA polymerases.

Studies of the Thermostability of DNA Polymerases. Proteins, at a concentration of at 0.1 mg/mL, in 25 μL of 20 mM Tris-HCl buffer (pH 8.0 at 25 °C), containing 1 M potassium glutamate (K-Glu) and 1 M betaine, were incubated in a PTC-150 minicycler (MJ Research) at fixed temperatures. Samples were removed at defined incubation times and assayed for primer extension activity with the standard substrate PTJ1.

Dependencies of the Rates and Processivity of Primer Extension Reactions on Temperature. The duplex for high-temperature primer extension assays was prepared by annealing a 5′-end, labeled with a fluorescein 40-nucleotide primer, with a 50-nucleotide template:

```
FL*
 |
GTGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT  -> extension
||||||||||||||||||||||||||||||||||||||||
CACACCACCACCACCACCACCACCACCACCACCACCAaaaaattttt      (PTJ2)
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The melting temperature of the duplex was found to be higher than 80 °C, even when no salts were added.

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DNA polymerase reaction mixtures contained 0.32 μM PTJ2, betaine and NaCl or K-Glu, as indicated, and appropriate amounts of DNA polymerases (final concentrations of 14−200 nM were chosen depending on the activity of the polymerases to produce similar initial rates of primer extension at 70 °C). The control (background) reaction mixtures contained all the components except the polymerases. The reactions were started by addition of PTJ2 to the reaction mixture preheated for 10 s at the specified temperatures. Primer extensions were conducted in the range of temperatures between 50 and 105 °C. For comparison, the reactions at 37−75 °C were conducted with the standard substrate PTJ1.

RESULTS

Construction of Chimeric Polymerases. DNA polymerase constructs used in this study are shown in Figure 1. We found previously that catalytic polymerase domains of thermostable DNA polymerases of both polA and polB structural families are able to produce functional hybrids with HhH domains of topoisomerase V from the hyperthermophile *M. kandleri*.[6] We suggested that structural domains with high internal stability could fold independently in chimeras, maintaining the high catalytic activity of such constructs. All previously analyzed hybrids included the entire C3 domain of Topo V, which contained HhH repeats H−L (10 HhH motifs). The C3 domain was previously produced as an individual protein, called Topo34, and its properties have been studied.[8] In active chimeras, the C3 domain is attached to the N-terminal or C-terminal end of the *Taq* polymerase catalytic domain; that is, it folds before or after the polymerase domain is formed. However, it is unknown if the presence, or integrity, of the C3 domain plays any role in the stabilization of the polymerase catalytic domains of the hybrid enzymes. Because of a strong positive charge the C3 domain carries on its C-termini (Figure 1A), it is particularly interesting to know if those positively charged amino acid residues could mediate domain interactions in chimeras. It is also important to see if the extreme thermal stability of the catalytic polymerase domains used in our previous experiments[6] could be a major factor for the successful formation of the active highly thermostable hybrid polymerases. To answer these and other questions, we have created three additional chimeras: (i) TaqTopoC1Δ, which encompasses the *Taq* polymerase domain and a domain formed by HhH repeats B−H of Topo V,[8] (ii) TaqTopoC2Δ, which was obtained from TaqTopoC2[6] by removing the last 16 amino acid residues that contribute to the very strong positive charge of the original chimera, and (iii) *Bst*C2, which has the C2 domain attached to the C-terminus of the polymerase domain of moderately thermolabile *Bst* DNA polymerase.

The successful expression of active and stable chimeric DNA polymerases in *E. coli*, taken together with previously published data,[6] provides strong support to our hypothesis of the independent folding of the catalytic and DNA binding HhH domains in natural DNA processing proteins.

As expected, *Taq* hybrids with the truncated tails have lower affinities for heparin Sepharose in comparison to those of the chimeras with the intact C3 domain. Surprisingly, the *Bst*C2 polymerase has a much lower affinity than *Bst*LF, indicating that a strong interdomain interaction hinders either part of the molecule from efficiently intermingling with heparin groups.

Increased Thermostability of DNA Polymerases with Attached Topo V HhH Domains. The remarkable thermostability of chimeric DNA polymerases was demonstrated for DNA polymerases of structural families A and B.[6] To further understand the mechanism of the stabilization of the polymerase catalytic domains at high temperatures facilitated by the introduced HhH domains, we designed new chimeras TaqTopoC1Δ and TaqTopoC2Δ, which are truncated versions of TaqTopoC1 and TaqTopoC2, respectively (see Materials and Methods and Figure 1B), and measured the time course of thermal inactivation of the chimeras and other DNA polymerases.

Analysis of the inactivation kinetics suggested a single-exponential decrease in the polymerase activities. The calculated rate constants of thermal inactivation for the studied DNA polymerases are listed in Table 1. As shown in the table, fusion of C1, C2, and C3 domains with the *Taq* polymerase catalytic domain, as well as fusion of the C2 domain with the *Pfu* DNA polymerase, maintains full enzymatic activity after incubation for 1 h at 100 °C, making the studied DNA polymerases as stable as the *Methanopyrus* PolB.

Table 1. Apparent Rate Constants for Thermal Inactivation of DNA Polymerases in the Presence of 1 M Potassium Glutamate and 1 M Betaine

DNA polymerase	k_{in} ± standard error ($\times 10^4$ s^{-1})
Incubation at 100 °C	
N-TopoTaq	<0.5
TaqTopoC1	<0.5
TaqTopoC1Δ	7.2 ± 0.8
TaqTopoC2	<0.5
TaqTopoC2Δ	1.7 ± 0.1
TaqTopoC3	<0.5
Taq pol	17.4 ± 2.1
Stoffel fragment	12.7 ± 1.2
Pfu pol	1.0 ± 0.2
*Pfu*C2	<0.5
Mka PolB	<0.5
Incubation at 95 °C	
*Bst*LF pol	699.9 ± 57.1
*Bst*C2	441.7 ± 15.2

Figure 2A displays the thermal inactivation of TaqTopoC1Δ and TaqTopoC2Δ at 100 °C in the presence of 1 M potassium glutamate and 1 M betaine; also, data for thermal inactivation of TaqTopoC2, *Taq* polymerase, and the Stoffel fragment are shown for comparison. Clearly, trimming the C-terminal sequences reduces the thermostability of the chimeric polymerases. It is interesting that while the entire C3 domain alone, being attached to either terminus of the Stoffel fragment (in chimeras TaqTopoC3 and N-TopoTaq), stabilizes the *Taq* polymerase catalytic domain for at least 1 h at 100 °C,[6] the removal of as many as 10 C3 HhH repeats from TaqTopoC1Δ does not completely abolish the aquired stability. The residual thermostability is provided by the remaining HhH repeats of domains B−H. As we suggested previously,[6] this effect of thermostabilization can be specifically attributed to the HhH repeats. In contrast, the natural exonuclease domains in *Taq* polymerase do not provide any stabilization (Figure 2A and Table 1). It is especially important that the effect of domains B−H not be associated with the strong positive charge residing in the C3 domain, as domains B−H should have overall negative charge at neutral and alkaline pH (Figure 1A). Furthermore, this observation is additionally supported by data on the thermal stability of TaqTopoC2Δ, as the removal of the

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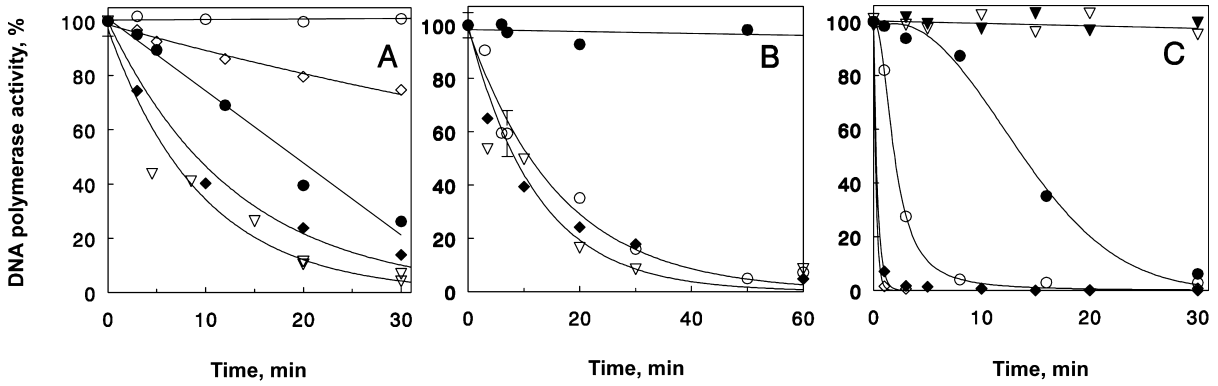

Figure 2. Thermostability of chimeric DNA polymerases (see Materials and Methods). (A) Stability of Taq polymerase chimeras with truncated C-terminal Topo V tails at 100 °C in the presence of 1 M potassium glutamate and 1 M betaine: (○) TaqTopoC2, (●) TaqTopoC1Δ, (◇) TaqTopoC2Δ, (▽) *Taq* polymerase, and (◆) Stoffel fragment. (B) Effect of NaCl on the thermostability at 100 °C: (○) N-TopoTaq in 0.5 M NaCl, (●) N-TopoTaq in 0.5 M NaCl and 1 M betaine, (▽) *Taq* polymerase, and (◆) Stoffel fragment. (C) Inactivation of the large fragment of *Bst* DNA polymerase and the *Bst*C2 chimera at 75 °C [(▽) *Bst*LF and (▼) *Bst*C2], 85 °C [(○) *Bst*LF and (●) *Bst*C2], and 95 °C [(◇) *Bst*LF and (◆) *Bst*C2].

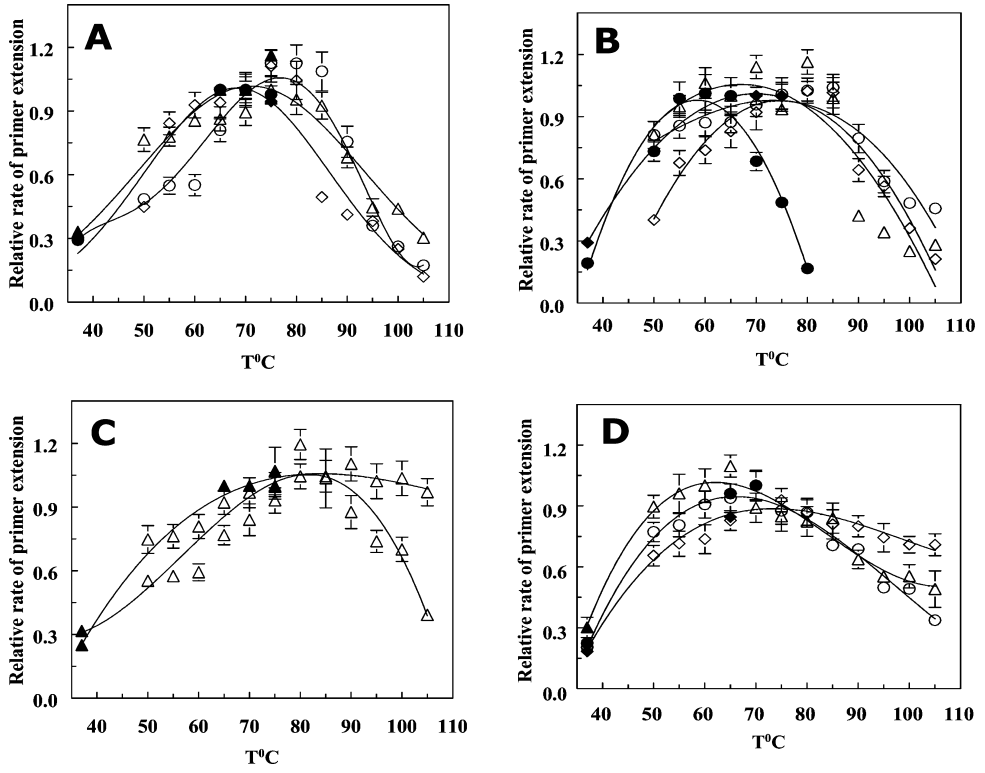

Figure 3. Dependencies of DNA polymerase activity on temperature. The empty symbols refer to the primer extension experiments conducted with PTJ1 substrate; the results obtained with PTJ2 are shown with filled symbols (see Materials and Methods). (A) (○, ●) *Taq* polymerase, (△, ▲) Stoffel fragment, and (◇, ◆) KlenTaq. (B) (○, ●) N-TopoTaq in potassium glutamate, (△) N-TopoTaq in NaCl, and (◇, ◆) TaqTopoC3. (C) *Taq* chimeras with C1 (△, ▲) and C2 (▽, ▼) tails. (D) *M. kandleri* polB av19 (○, ●), *Pfu* DNA polymerase (△, ▲), and PfuC2 (◇, ◆). All kinetic measurements were taken in triplicate, and the error bars represent standard deviations for each experimental value.

16 terminal amino acids should make neutral the net charge of the HhH tail at low alkaline pH. Nonetheless, the remaining Topo V domains still exert a very strong effect on thermostability.

Thus, it appears that the thermal stabilization of chimeric polymerases occurs due to charge-independent interactions of the polymerase domain and HhH domains. It is interesting that at 100 °C the thermostabilities of the *Taq* polymerase and the Stoffel fragment are almost the same in 0.5 M NaCl and in 1 M potassium glutamate with 1 M betaine. In contrast, the stabilizing effect of the C3 domain in N-TopoTaq completely disappears if K-Glu is replaced with 0.5 M NaCl, and the stability of N-TopoTaq does not differ from that of the catalytic domain alone (Figure 2A,B). However, the addition of 1 M betaine restores the stabilization provided by the C3 domain (Figure 2B). A similar decrease in thermostability was observed in NaCl-containing buffers for TaqTopoC1-C3 even at 95 °C (data not shown).

Figure 2C demonstrates the thermostability of the *Bst*LF polymerase with the attached Topo V's HhH C2 domain. The

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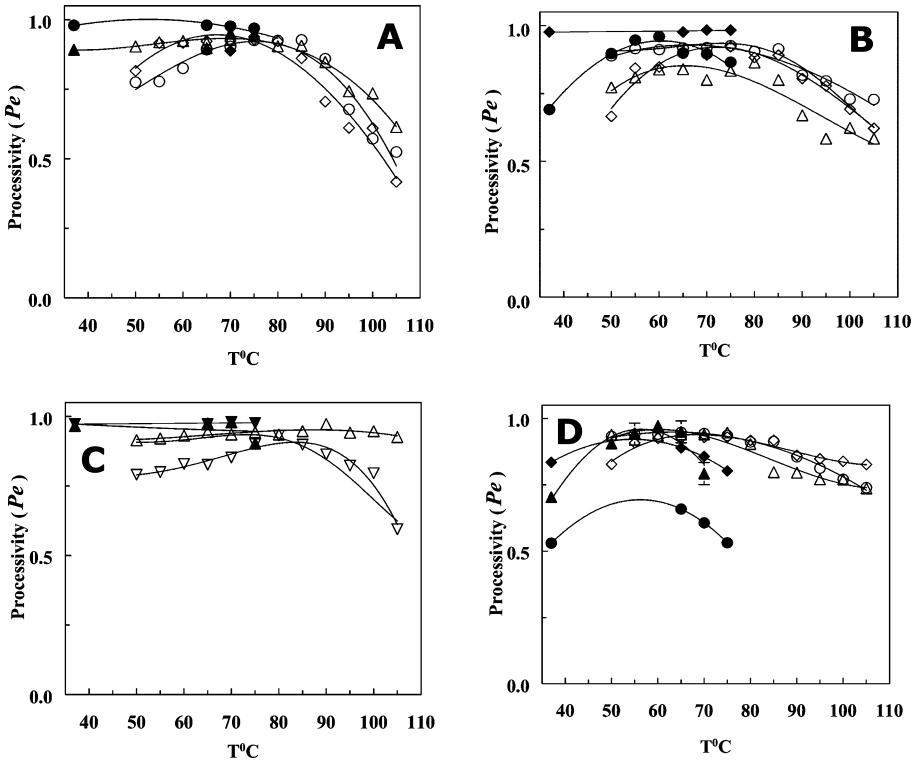

Figure 4. Dependencies of DNA polymerase processivity on temperature. (A) [(○) PTJ1, (●) PTJ2] *Taq* polymerase, [(△) PTJ1, (▲) PTJ2] Stoffel fragment, and [(◇) PTJ1, (◆) PTJ2] KlenTaq. (B) [(○) PTJ1, (●) PTJ2] N-TopoTaq in potassium glutamate, [(△) PTJ1] N-TopoTaq in NaCl, and [(◇) PTJ1, (◆) PTJ2] TaqTopoC3. (C) *Taq* chimeras with C1 [(△) PTJ1, (▲) PTJ2] and C2 [(▽) PTJ1, (▼) PTJ2] tails. (D) *Mka* polB av19 [(○) PTJ1, (●) PTJ2], *Pfu* DNA polymerase [(△) PTJ1, (▲) PTJ2], and PfuC2 [(◇) PTJ1, (◆) PTJ2]. All kinetic measurements were taken in triplicate, and the error bars represent standard deviations for each experimental value.

half-life of the chimeric protein increases almost 8-fold, as compared to that of the *Bst*LF polymerase alone. The effect can be seen up to 95 °C (Figure 2C and Table 1), with estimated half-lives of 10 and 15.7 s for *Bst*LF and *Bst*C2, respectively. It is worth noting, however, that the seemingly universal stabilization of different DNA polymerases domains by the fused Topo V HhH repeats is limited by the stability of polymerase catalytic domains.

DNA Synthesis by Chimeric Polymerases over a Broad Range of Temperatures. Using a specially designed duplex substrate with a high melting temperature (PTJ2), we studied primer extensions by *Taq* and *Pfu* DNA polymerases, fragments of *Taq* polymerase, and chimeric polymerases at temperatures from 37 to 105 °C (Figure 3). Figures 3−5 show relative initial rates of DNA synthesis, apparent rates of substrate binding, and processivities versus temperature for different DNA polymerases measured in the kinetic assays at 37−105 °C. The catalytic domains of both *Taq* and *Pfu* DNA polymerases conduct DNA synthesis at least at 105 °C with nearly the same efficiency as at lower temperatures (Figures 3C,D and 4C,D). At low temperatures (<70 °C), the initial rates measured with PTJ1 display virtually the same dependencies of relative activity on temperature, although the absolute values of the rates are different (e.g., at 70 °C, the initial rate of extension of 0.32 μM PTJ2 is ∼2 times higher than that of PTJ1 at the same concentration).

As shown in Figure 3 (panels G−I), the rate of primer extension for PTJ2 with TaqTopoC1 virtually does not change in the range of 75−105 °C. In addition, it turned out that more than 96% of the substrate can be elongated if longer incubation with the enzyme is allowed. This implies that the melting rates of PTJ2 are much lower than the rates of primer extension at all temperatures of the assays. Therefore, substrate melting does not affect the measurement of the initial rates, particularly when the reactions get started by adding duplex substrates to the reaction mixtures containing DNA polymerases equilibrated at the temperatures of the assay (see Materials and Methods). However, this was not the case with PTJ1 (Figure 3D), as melting of this substrate (T_m = 73.2 ± 0.4 °C) prevents its efficient extension at high temperatures.

The temperature profiles of the polymerization rates of N-TopoTaq and TaqTopoC3 (Figure 3B) above 70 °C are very similar, and they do not differ significantly from those for the catalytic Stoffel fragment, *Taq* polymerase, or KlenTaq (Figure 3A). Therefore, it appears that the additional domains of these polymerases are not essential for the high-temperature DNA synthesis by the *Taq* polymerase catalytic domain. Also, because primer extension by *Taq* polymerase and its fragments is performed in 50 mM KCl (higher salt concentrations inhibit these polymerases) and the reactions with the chimeras are conducted in the presence of 0.25 M salts and 1 M betaine, one may conclude that heat inactivation of the *Taq* polymerase catalytic domain is not sensitive to the salt concentration. Accordingly, only minor differences are observed between the curves for N-TopoTaq in the presence of 0.25 M NaCl and 0.25 M K-Glu (Figure 3B), although the absolute values of the initial rates are higher in K-Glu.

An effective high-temperature DNA synthesis (up to 105 °C) by chimeric polymerases was observed only for C1 and C2 constructs (Figure 3C,D). The effect of the C1 and C2 domains is seen in cases of chimeras containing either *Taq* or *Pfu* catalytic domains, which suggests that other thermostable

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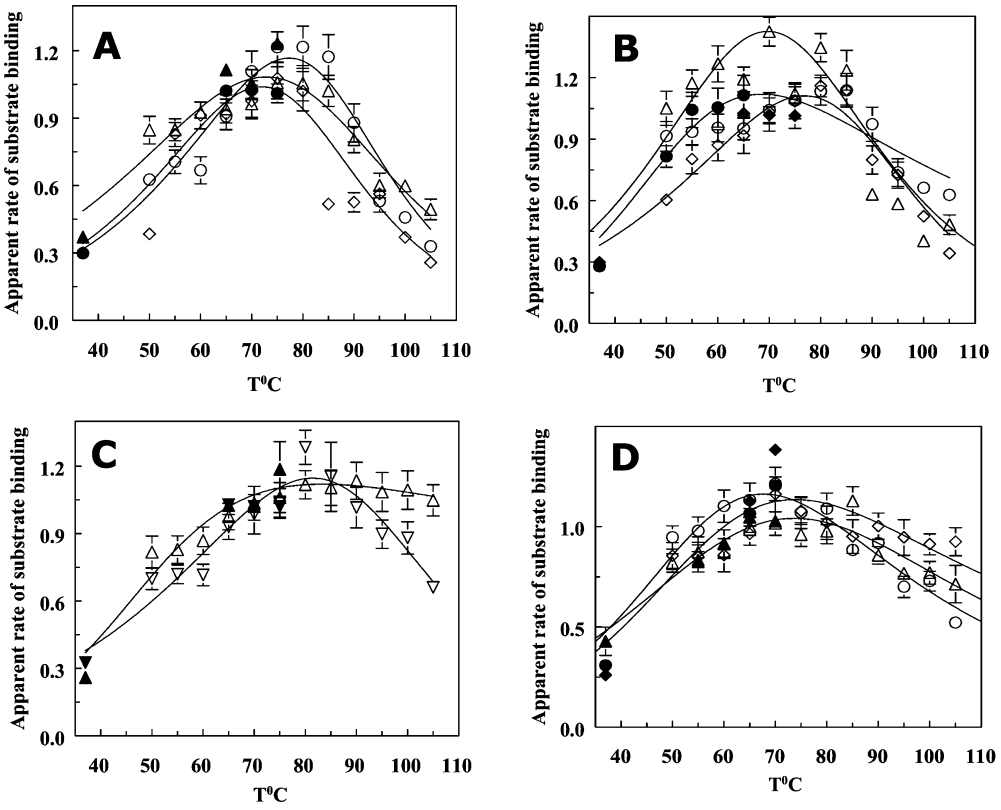

Figure 5. Dependencies of the apparent rate of substrate binding by DNA polymerases on temperature. (A) [(○) PTJ1, (●) PTJ2] *Taq* polymerase, [(△) PTJ1, (▲) PTJ2] Stoffel fragment, and [(◇) PTJ1, (◆) PTJ2] KlenTaq. (B) [(○) PTJ1, (●) PTJ2] N-TopoTaq in potassium glutamate, [(△) PTJ1] N-TopoTaq in NaCl, and [(◇) PTJ1, (◆) PTJ2] TaqTopoC3. (C) *Taq* chimeras with C1 [(△) PTJ1, (▲) PTJ2] and C2 [(▽) PTJ1, (▼) PTJ2] tails. (D) *Mka* polB av19 [(○) PTJ1, (●) PTJ2], *Pfu* DNA polymerase [(△) PTJ1, (▲) PTJ2], and PfuC2 [(◇) PTJ1, (◆) PTJ2]. The solid lines are theoretical curves calculated from eq 7.

polymerases could be adopted for DNA synthesis at extremely high temperatures, as well.

The profiles at lower temperatures (37−70 °C) in Figure 3 show decreased activities for *Taq* polymerase, KlenTaq, TaqTopoC2, and TaqTopoC3 with respect to the Stoffel fragment, suggesting that there are domain interactions that emerge at lower temperatures and interfere with DNA synthesis.

The data in Figures 3 and 4 allow for evaluation of apparent rates of substrate binding [$V_{b,app}$ (see the Supporting Information for details)] shown in Figure 5A−D. Figure 5 thus demonstrates that the drop in the rates of substrate binding rather than the decrease in processivity (P_e) (Figure 4A−D) is the major contributor to the deceleration of the polymerization by the DNA polymerases at high temperatures.

Analysis of Substrate Binding by DNA Polymerases at High Temperatures. The analysis of substrate binding was conducted using a general model that includes temperature-dependent reversible isomerization of DNA polymerases (see the Supporting Information for details). Accordingly, the dependencies of $V_{b,app}$ on temperature for all polymerases shown in Figures 5A−D can be approximated by theoretical bell-shaped curves predicted by this model. Table 2 summarizes values for the pre-exponential factor and activation energies of binding of the substrate to DNA polymerases and the enthalpy and entropy changes of the conformational transitions (see the Supporting Information).

Fusion of various HhH domains with DNA polymerases causes relatively small changes in the parameters of a DNA binding reaction; e.g., values of activation energies for the formation of complexes with PTJ are almost the same for Stoffel fragment, *Taq* polymerase, and the chimeric TaqTopoC2 and TaqTopoC3. This suggests that the additional Topo V domains at the C-termini of the *Taq* polymerase catalytic domain do not interfere with the initial binding of the duplex substrates to the polymerase active site. Accordingly, it implies

Table 2. Values of the Pre-Exponential Factor, Activation Energy for Binding of the Duplex Substrate to DNA Polymerases, and the Enthalpy and Entropy Changes for the High-Temperature Reversible Conformational Transition of DNA Polymerases That Eliminates Binding of the Substrate

DNA polymerase	A (M^{-1} s^{-1})	E_a (kJ/mol)	$\Delta S^{\ddagger}$ (J/K)	$\Delta H°$ (kJ/mol)	$\Delta S°$ (J/K)
N-TopoTaq[a]	1.63×10^{10}	3.69	−2.89	4.99	15.10
N-TopoTaq[b]	4.40×10^{6}	4.01	−6.79	6.41	19.16
TaqTopoC1[a]	1.86×10^{4}	3.83	−9.39	4.17	12.85
TaqTopoC2[a]	4.49×10^{5}	1.92	−7.88	5.70	15.78
TaqTopoC3[a]	9.47×10^{5}	1.82	−7.52	7.02	19.53
Taq pol[c]	7.44×10^{5}	1.95	−7.64	7.13	19.87
Stoffel fragment[c]	6.09×10^{4}	1.94	−8.83	5.42	15.39
KlenTaq[c]	3.06×10^{6}	2.21	−6.97	6.58	18.76
Pfu pol[a]	1.61×10^{9}	3.28	−3.99	5.09	15.21
PfuC2[a]	3.17×10^{9}	3.35	−3.67	4.40	13.23
Mka PolB[a]	2.83×10^{5}	2.05	−8.10	3.83	11.11

[a]In 250 mM potassium glutamate. [b]In 250 mM sodium chloride. [c]In 50 mM potassium chloride.

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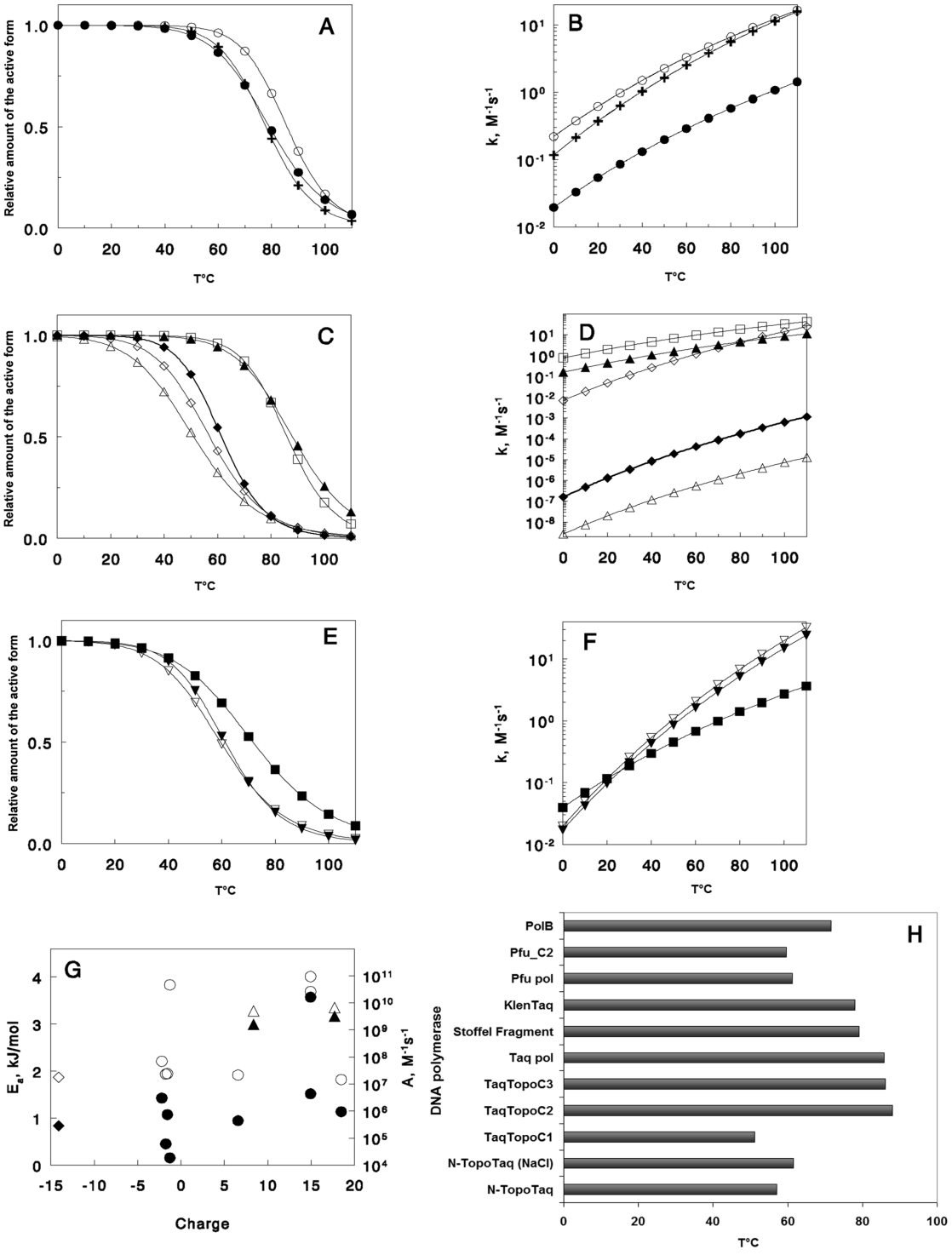

Figure 6. Theoretical values calculated from the parameters of Scheme 2 of the Supporting Information. The level of accumulation of the inactive conformations with temperature increases, and dependencies of DNA binding rate constant on temperature for *Taq* polymerase and its fragments (A and B), chimeric polymerases with the *Taq* catalytic domain (C and D), and polB DNA polymerases (E and F): (○) *Taq* polymerase, (●) Stoffel fragment, (+) KlenTaq, (△) TaqTopoC1, (▲) TaqTopoC2, (□) TaqTopoC3, (◇) N-TopoTaq in potassium glutamate, (◆) N-TopoTaq in NaCl, (▽) *Pfu* DNA polymerase, (▼) PfuC2, and (■) *Mka* polB AV19. (G) Dependencies of the pre-exponential factor (filled symbols) and activation energy (empty symbols) of the Arrhenius equation for binding of PTJ to DNA polymerases[6] on the total charge of the protein molecules calculated at 75 °C: (○, ●) polymerases with the *Taq* catalytic domain, (△, ▲) polymerases with the *Pfu* catalytic domain, and (◇, ◆) *M. kandleri* polB av19. (H) Temperatures of half-inactivation for DNA polymerases (curves in panels A, C, and E).

that during the initial binding of the substrate to the native *Taq* polymerase, the N-terminal exonuclease domain has a remote position with respect to the catalytic domain, as it was revealed by structural analysis.[17] The slightly higher activation energy of the KlenTaq polymerase compared to those of the Taq polymerase and Stoffel fragment could indicate interference of the truncated exonuclease domain with substrate binding during the reaction. In contrast, both N-TopoTaq and

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TaqTopoC1 possess much higher activation energies. This could be a result of the localization of the Topo V C3 domain to be in the proximity of the polymerase active site, which would result in partial blockage of substrate binding. Thus, the efficient binding of the substrate likely requires a conformational change such as removing the interfering domains. These findings support our earlier suggestion[6] that in the TaqTopoC1 construct, the C3 domain could be in the proximity of the catalytic site because of the length of the C-terminal tail of the chimera.

It is interesting that in *Pfu*C2 DNA polymerase the C2 domain is positioned on the substrate binding side but does not alter the activation energy of substrate binding, which is already much higher than that for the *Taq* polymerase catalytic domain. It is possible that the high activation energy of binding reflects conformational changes in the *Pfu* catalytic domain similar to those in bacteriophage RB69 polB.[18,19] However, this is not necessarily a general property of polB catalytic domains, as the E_a of *M. kandleri* DNA polymerase is very similar to that of the *Taq* Stoffel fragment (Table 2).

Surprisingly, the empirical pre-exponential factor of the Arrhenius equation (see the Supporting Information) was found to have variations over a very wide range between different DNA polymerases (Table 2). The pre-exponential factor can be interpreted as a product of collision frequency and a steric factor of the reaction.[20] As the values of collision frequencies should be similar for the reactions of all studied DNA polymerases with the PTJs, the differences could be related to the efficiency of the collisions or the steric factor.

In Figure 6, both the values of E_a and the pre-exponential factors are plotted against the calculated charge of the enzymes at pH 6.5 (estimated pH of the reaction in Tris-HCl buffer at 75 °C). Although several groups of DNA polymerases have similar activation energies or empirical pre-exponential factors, those values do not correlate with the total charge of the molecules (Figure 6G). Therefore, the mechanism of electrostatic enhancement of diffusion-controlled association[21] suggesting the reduction of E_a for binding substrates as a result of charge interactions does not play a significant role in binding of PTJs to DNA polymerases in our case. Also, for charged molecules, the theory of diffusion-controlled association predicts that the electrostatic enhancement and, consequently, the rate of substrate binding would depend on the total ionic strength of the solution. However, in the presence of NaCl, the pre-exponent factor for N-TopoTaq was almost 4 orders of magnitude lower than in potassium glutamate at approximately the same ionic strength, with an only slightly higher E_a (Table 2). This result is in agreement with our previous finding that chloride ions can specifically block positive charges on HhH domains of Topo V[6] that interact with DNA, rather than decrease the rate of DNA binding by changing the total charge distribution of the molecules.

In terms of transition state theory, the steric factor, which is included in the pre-exponential factor, can be related to the entropy of activation of substrate binding. As all studied polymerases have strong negative values of the entropy of activation, $\Delta S^{\ddagger}$ (Table 2), it appears that the formation of polymerase−PTJ complexes renders structures of the proteins and PTJ more compact than in the solution before the interaction.

The calculated rate constants are presented in panels B, D, and F of Figure 6. As compared to *Taq* polymerase and KlenTaq, the Stoffel fragment has ∼10-fold lower rate constants of association with the PTJ because of the low steric factor of complex formation (Figure 6B). These observations suggest an important role of the *Taq* polymerase 5′-exonuclease domain for providing efficient DNA association with the polymerase site, e.g., by offering additional DNA binding groups for favorable orientation of the substrate. Also, TaqTopoC2 and TaqTopoC3 have rate constants similar to that of the *Taq* polymerase, apparently because of the very close values of the pre-exponential factors and activation energies (Table 2). Therefore, Topo V HhH domains could substitute for the original *Taq* polymerase 5′-exo domain to provide the proper orientation of the DNA substrate for faster binding to the polymerase site.

Fusion of the C3 domain to the N-terminus of the *Taq* polymerase catalytic domain resulted in large changes in both the pre-exponential factor and the activation energy, with respect to those of the Stoffel fragment alone (Table 2, N-TopoTaq without added NaCl). Nevertheless, the rate constants for N-TopoTaq and the Stoffel fragment behaved very similarly (compare curves in Figure 6B vs those in Figure 6D), because the ∼1000-fold increase in the pre-exponential factor in the Arrhenius equation for N-TopoTaq compensated for the increase in activation energy. In contrast, the addition of 0.25 M NaCl, which significantly weakens the interaction of DNA with HhH domains,[6] decreases the pre-exponential factor and the corresponding rate constant of binding ∼10000-fold (Figure 6D), as compared to that of the Stoffel fragment.

The extremely low pre-exponential factor found for TaqTopoC1, evidently, originates from poor steric conditions for DNA binding. In combination with a very high activation energy [as in the case of N-TopoTaq (Table 2)], it produces binding rate constants $\sim 10^6$ times lower than those of the Stoffel fragment.

PolB DNA polymerases demonstrate a temperature profile of the binding rate constants comparable to that of *Taq* polymerase (Figure 6F). Interestingly, fusion of the C2 domain to the C-terminus of the catalytic domain of *Pfu* DNA polymerase does not produce any significant differences either in the pre-exponential factor or in the activation energy. It appears that the interaction of DNA with the HhH domains during the initial substrate binding does not improve the orientation of the substrate with respect to the polymerase DNA-binding site, or such interaction occurs only after the binding of DNA to the polymerase site. The parameters for binding of DNA to *M. kandleri* PolB were similar to those of TaqTopoC2 (Table 2 and Figure 6F).

The reversible conformational transition of DNA polymerases makes the binding of the PTJ substrate to the polymerase catalytic domain at high temperatures inefficient. This transformation is controlled by the values of the changes in enthalpy and entropy. As both the enthalpy and entropy changes are positive, it suggests that the polymerases adopt less compact and less stable conformations. However, because the changes in enthalpy and entropy have the opposite effect on the transformation, they determine a variety of the dependencies of the equilibrium (Scheme 2 of the Supporting Information) on temperature. Figure 6 (A, C, and E) shows the calculated relative amounts of substrate binding polymerase conformations versus temperature. Surprisingly, such transitions occur at rather low temperatures: for the majority of the polymerases in this study, the temperature of half-transformation is below 80 °C (Figure 6H). Moreover, the *Pfu* catalytic domain shows a transformation temperature much lower than that of the *Taq*

polymerase. Both C2 and C3 domains on the C-termini of the Stoffel fragment as well as the whole *Taq* exo domain of the original *Taq* polymerase seem to stabilize the active conformation. However, the transformation into an inactive form occurs at very low temperatures in the case of N-TopoTaq and TaqTopoC1. As this effect is not observed for *Taq* polymerase, its fragments, or other *Taq* hybrids, it can be attributed to the interference of the C3 domain with PTJ binding in these chimeras.

DISCUSSION

In light of this work, previous data revealing the increase in the thermostability of the hybrids[6] become even more intriguing, as they demonstrate direct interactions of the polymerase domains and HhH domains. Our modeling of Topo V HhH domains in chimeras,[6] which is in agreement with the crystal structure of several Topo V HhH domains,[22] suggests that HhH repeats B–L are folded into individual structures of similar shape connected with each other by flexible linker sequences. Therefore, the thermo-stabilizing effect in chimeras could occur either because of wrapping of the flexible HhH tail around the polymerase domain, thereby restricting its conformational mobility, or through some specific contacts between the polymerase domain and C3 domain, which has its own stable structure.[8] Comparison of the thermostabilities of chimeras (Figure 2) points to a mechanism of thermo-stabilization that involves specific interactions of the polymerase catalytic domains with the HhH C3 domain. This conclusion is supported by the fact that the incorporation of the C3 domain alone suffices to produce the largest increase in the chimera's thermostability, while truncations of the C3 domain (Figure 1) have the strongest effect on the stability of the hybrids at high temperatures. The specific interdomain contacts in chimeras are likely produced by electrostatic interactions (e.g., between positively charged Topo V domain L and negatively charged N-termini in catalytic fragments of polA or thumb domains in polB), because chloride ions, which are known to impair charge-directed interactions of the C3 domain, eliminate the improved stabilization.

The ability to increase the polymerase thermal stability is not limited to the C3 domain because other hybrid DNA polymerases with the C1Δ, C2, and C2Δ HhH domains are all more thermostable than the Stoffel fragment alone (Figures 1 and 2). It is interesting in this regard that the exonuclease domain of the entire *Taq* polymerase, which also contains a single copy of the HhH fold, does not contribute to the thermostability of the polymerase domain. Unlike the highly temperature resistant HhH domains of Topo V, the 5′-exo *Taq* polymerase domain melts separately at a lower temperature (89 °C) than the *Taq* polymerase catalytic domain (97–100 °C);[23] thus, it cannot stabilize the catalytic domain at high temperatures.

Studies of the polymerase reaction at very high temperatures (>80 °C) reveal several effects of HhH domains on DNA synthesis. The most interesting information was obtained while analyzing the effect of temperature on elongation rates that, under the selected reaction conditions, reflects rates of binding of the DNA substrates to DNA polymerases and formation of the productive complexes for DNA elongation. We found that a decrease in the rate of DNA synthesis by DNA polymerases at high temperatures could be attributed to the reversible high-temperature isomerization of the DNA polymerase catalytic domains. Another important parameter of the binding reaction is the rate of formation of the productive complexes upon association with a DNA substrate with the fraction of DNA polymerase molecules, which are present in active conformations. Despite the highly negative charge of DNA substrates, the mechanism of electrostatic enhancement of diffusion-controlled association[21] does not play a significant role in the initial binding of the studied polymerases to DNA. Rather, the position of the additional charged domain is important for the mechanism of binding. For example, the calculated charge on a TaqTopoC3 molecule at 75 °C (+18.46) is higher than that of N-TopoTaq (+14.95). However, in 0.25 M K-Glu, the pre-exponential factor for N-TopoTaq is ∼17000 times higher than that for TaqTopoC3, suggesting that every collision of DNA with N-TopoTaq has 17000 more chances to form the productive complex than collision with TaqTopoC3, if DNA and N-TopoTaq molecules have sufficient energies. Nonetheless, the activation energy for the binding reaction of DNA with N-TopoTaq is much higher than that of the binding reaction for TaqTopoC3 (Table 2), so that a significantly smaller fraction of collisions with properly oriented N-TopoTaq molecules resulted in DNA binding. Moreover, the interaction of the N-TopoTaq HhH domains with the catalytic polymerase domain in the absence of the bound substrate decreases the number of N-TopoTaq molecules in a conformation suitable for DNA binding (Figure 4A,C). As a result, the overall rate of DNA synthesis by N-TopoTaq is much lower than the rate of synthesis by TaqTopoC3.

Data in Table 2 show that for the majority of the DNA polymerases of this study, the activation energy increases alongside the pre-exponential factor, pointing to a linear correlation between E_a and log A. In this case, the Eyring equation for the rate constant[20] suggests a constant value of the Gibbs activation energy among various DNAs. Figure 7 shows

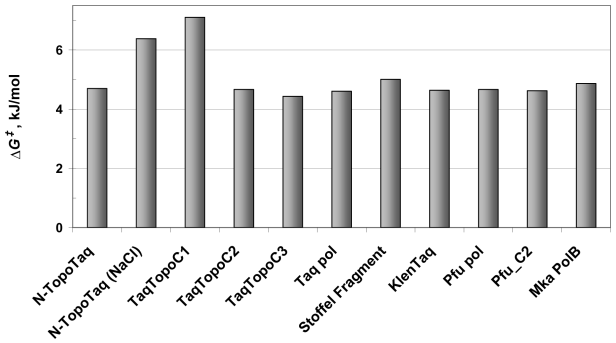

Figure 7. Values of the Gibbs energy of activation for binding of PTJ1 to various DNA polymerases.

that the values of the Gibbs energy of activation are very similar for DNA polymerases of various classes and with different HhH domains. Therefore, in the reaction of binding DNA and formation of the productive complex (E + S → $ES^{‡}$ → ES_0), the $ES^{‡}$ transition states of diverse DNA polymerases should have similar energies and geometries. Evidently, the latter is a basic property of intact DNA polymerase domains, which is not changed in the majority of the hybrid enzymes. Two exceptions, N-TaqTopo (NaCl) and TopoTaqC1, show that the transition state can be affected by unfavorable positioning of HhH domains.

Moreover, the values of the Gibbs activation energy do not depend on the overall charges of the DNA polymerases, suggesting that the transition state is not affected by the charge

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of DNA polymerases either, and the binding of the substrate to the active site is not controlled by diffusion.

The HhH tails in the chimeras can stabilize the enzyme complexes with the DNA substrate, as is noted by the processivity plots (Figure 4). It is interesting that all Topo V HhH−*Taq* chimeras used in this study demonstrate similar low levels of stabilization, except for TaqTopoC1, whose value significantly exceeds those of the others. As all polymerase complexes with a primer−template junction substrate are different with respect to salt resistance,[6] this suggests that salt-induced dissociation and heat-induced dissociation of the same complexes have different mechanisms and involve different steps.

HhH domains are widely present in enzymes that act on nucleic acids and can be catalytically involved with base excision repair.[7,24] Little is known about the functional interaction of HhH domains with other domains of DNA processing proteins. An important role of HhH domains in contact between the human pol λ and PCNA has been reported.[25] Using artificial hybrid proteins,[6] we demonstrated other types of interdomain interactions involving the HhH motifs. In a recent paper,[10] Salas and co-workers reported the successful fusion of HhH domains from the *M. kandleri* Topo V with ϕ29 DNA polymerase, which brings about a general improvement in amplification reactions conducted by the hybrid enzyme. Although the authors do not analyze the mechanism of domain interaction in the chimera, they assume that the major cause of the enhancement of DNA synthesis is the improved binding of the hybrid polymerase to the DNA template. However, it is also possible that the reported improvement results from the direct interaction of the HhH domains with the polymerase.

■ ASSOCIATED CONTENT

Supporting Information

Detailed information such as a general model for substrate binding by DNA polymerases and kinetic schemes used to describe the effects of temperature on duplex binding and processivity of DNA synthesis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Mka polB, *M. kandleri* DNA polymerase of family B; *Bst* DNA polymerase, *B. stearothermophilus* DNA polymerase of family A; LF *Bst* polymerase, large fragment of *B. stearothermophilus* DNA polymerase of family A; *Pfu* polB, *Pyrococcus furiosus* DNA polymerase of family B; HhH, helix−hairpin−helix DNA binding; PTJ, primer−template junction; $V_{b,app}$, apparent rate of substrate binding; P_e, processivity equivalence parameter; p_0, microscopic processivity parameter.

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