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Accurate DNA synthesis by *Sulfolobus solfataricus* DNA polymerase B1 at high temperature

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Abstract The accuracy of DNA synthesis by DNA polymerase B1 from the hyperthermophilic archaeon *Sulfolobus solfataricus* (*Sso* pol B1) at near the physiological temperature was investigated using M13-based mutational assays. *Sso* pol B1 showed replication fidelity similar to or higher than most viral, bacterial, and eukaryotic replicases. The fidelity of the enzyme was about three times as high at 70°C as at 55°C. Approximately two-thirds of the errors made by the enzyme were single-base substitutions, of which 58% were C → T transition. Frameshift mutations, mostly resulting from single-base deletions, accounted for 19% of the total errors. An exonuclease-deficient mutant of *Sso* pol B1 was three times as mutagenic as the wild-type enzyme, suggesting that the intrinsic proofreading function contributed only modestly to the fidelity of the enzyme. Kinetic assays showed that the frequencies of all possible misincorporations by an exonuclease-deficient triple-point mutant of *Sso* pol B1 ranged from 5.4×10^{-5} to 4.6×10^{-4}. The high fidelity of this enzyme in DNA synthesis was based primarily on K_m difference rather than V_{max} difference. These properties of *Sso* pol B1 are consistent with the proposed role of the enzyme as a replicase in *S. solfataricus*.

Keywords Archaea · *Sulfolobus solfataricus* · DNA polymerase · Fidelity · Mutation spectrum · Steady-state kinetics

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Introduction

Archaea have evolved a simplified version of the eukaryotic DNA replication machinery. Extensive biochemical and structural studies of archaeal DNA replication proteins in the past decade have shed significant light on the diversity and evolution of DNA replication mechanisms, providing important clues to the adaptation of DNA replication to extreme environmental conditions such as high temperature (Barry and Bell 2006; Grabowski and Kelman 2003).

Almost all genome-sequenced archaea, including members of the Crenarchaeota and the Euryarchaeota, encode one or more B-family DNA polymerases that are related to polymerases in the primary eukaryotic replisome (including pols α, δ, and ε). *Sulfolobus solfataricus*, one of the most intensively studied hyperthermophilic Crenarchaea, encodes three B-family DNA polymerases (pols B1, B2, and B3) as well as a Y-family DNA polymerase IV (Dpo4) (She et al. 2001). The organism grows optimally at ~80°C and pH 2–4. Of the three family B polymerases, only *Sso* pol B1 has been investigated (Lou et al. 2004a; Pisani et al. 1998). *Sso* pol B1 contains both 5′–3′ DNA polymerase and 3′–5′ exonuclease activities. Its 3′–5′ exonuclease activity is capable of detecting and removing misincorporations before chain extension (Lou et al. 2004b). Both polymerase and exonuclease activities of

Sso pol B1 are modulated by the *Sulfolobus* chromatin protein Sul7d at high temperatures (Lou et al. 2004a). The crystal structure of *Sso* pol B1 reveals a structural arrangement similar to other B-family polymerases in that they all contain an N-terminal 3′–5′ 'proofreading' exonuclease domain and a C-terminal 'right-hand' 5′–3′ polymerase domain (Savino et al. 2004). *Sulfolobus* utilizes a proliferating cell nuclear antigen (PCNA) heterotrimer as the sliding clamp and a replication factor C (RFC) complex as the clamp loader. A mixture of PCNA and RFC, or PCNA alone, was found to be able to stimulate DNA synthesis activity by *Sso* pol B1 in vitro (De Felice et al. 1999; Dionne et al. 2003). These findings support the notion that *Sso* pol B1 is a replicative DNA polymerase in *Sulfolobus*.

Replicative DNA polymerases from Bacteria and Eukarya are known for their high fidelity in DNA replication. In a recent pre-steady-state kinetic study, *Sso* pol B1 was shown to be capable of faithful DNA synthesis at 37°C in vitro (Zhang et al. 2009). In this study, we determined the fidelity of *Sso* pol B1 at near the optimal temperature for the growth of the organism and the mutation spectrum of the enzyme. We show that the fidelity of *Sso* pol B1 increased as temperature was raised to near the physiological temperature. Further, errors made by the enzyme during DNA synthesis were dominated by single point nucleotide substitutions, among which over half were the C → T transition.

Materials and methods

Bacteria and phage

Escherichia coli strains NR9099, MC1061 and CSH50, bacteriophage M13mp2KE were generous gifts from Thomas Kunkel (NIH, USA).

Primed templates

The following oligonucleotides were synthesized and purified by gel electrophoresis: primer p21 (5′-CGC AGC CGT CCA ACC AAC TCA), p42 (5′-CAG TGA ATT CGA GCT CGG TAC CCG GGG ATC CTC TAG AGT CGA), p30 (5′-CAG TGA ATT CGA GCT CGG TAC CCG GGG ATC), template t76 (5′-TTT TTA CGC CAA GCT TGC ATG CCT GCA GGT CGA CTC TAG AGG ATC CCC GGG TAC CGA GCT CGA ATT CAC TGT TTT T) and t41 (5′-GGA CGG CAT TGG ATC GAC GNT GAG TTG GTT GGA CGG CTG CG), where N is G, A, C, and T (Lou et al. 2004a; Zhang et al. 2009). The primers were labeled at the 5′ end with T4 polynucleotide kinase and purified using a G25 microspin column (Amersham Pharmacia Biotech, Piscataway, USA). A primed template was prepared in an annealing reaction in which a primer and a template were mixed at a molar ratio of 1:1.3 in 50 mM Tris–Cl, pH 7.5, and 100 mM NaCl, heated at 95°C for 5 min and cooled down to room temperature.

Proteins

Recombinant wide-type *Sso* pol B1 was prepared as described previously (Lou et al. 2004a). An exonuclease-deficient mutant of *Sso* pol B1 (D231A) was constructed using the QuickChange™ XL site-directed Mutagenesis kit (Stratagene, La Jolla, USA) for M13mp2-based fidelity assays. Expression vector for *Sso* pol B1 exo$^-$, an exonuclease-deficient triple-point mutant of *Sso* pol B1 (D231A, E233A, and D318A), was a generous gift from Zucai Suo (Ohio State University, USA). The triple-point mutant was used in the steady-state kinetic assays. Expression and purification of mutant proteins were carried out as described for wild-type *Sso* pol B1. Native Ssh7 proteins were purified as described previously (Mai et al. 1998). Protein concentrations were determined by the Lowry method using bovine serum albumin (BSA) as the standard (Dawson and Heatlie 1984).

M13-based mutation assays

Gapped M13mp2 DNA substrates containing a 407-nucleotide gap between −216 and +191, where +1 denotes the first transcribed nucleotide of the *lacZα* coding sequence, were prepared as described previously (Bebenek and Kunkel 1995). A standard gap-filling reaction (20 µl) contained wild-type *Sso* pol B1 or exonuclease-deficient mutant D231A (200 nM) and the gapped substrate (250 ng) in 50 mM Tris–HCl, pH6.5 or pH8.0, 2 mM β-mercaptoethanol, 100 µg/ml BSA, 4 mM $MgCl_2$, or $MnCl_2$ and 1 mM dNTPs (Amersham Pharmacia Biotech, Piscataway, USA). Ssh7 (25 µM) was added when indicated. The reaction was incubated at a specified temperature for 5 min and terminated with the addition of EDTA (50 mM).

Both forward and reversion mutation assays were performed as described (Bebenek and Kunkel 1995). For forward mutation assays, products of the gap-filling reactions were introduced into *E. coli* MC1061 cells by electroporation. Cells are plated onto indicator agar plates. After incubation for overnight at 37°C, dark blue (wild-type) and colorless or light blue (mutant) plaques were scored and the frequency of *lacZα* mutants was calculated as described previously (Bebenek and Kunkel 1995). Single-stranded phage DNA was isolated from independent mutant plaques, and the *lacZα* gene was sequenced to determine specific nucleotide changes. The number of mutant plaques relative to the total number of plaques scored (mutant frequency, MF) reflected the error rate, which was calculated as follows: $ER = [(N_i/N_t) \times MF]/$

($D \times 0.6$) (Shcherbakova et al. 2003), where N_i is the number of mutations of a particular type, N_t is the total number of mutants analyzed, MF is the frequency of *lacZα* mutants, D is the number of detectable sites for a particular type of mutation, and 0.6 is the probability that a polymerase error in the newly synthesized minus strand will be expressed in *E. coli* (Bebenek and Kunkel 1995).

For reversion mutation assays, base substitution errors occurring in the gap-filling DNA synthesis were measured using M13mp2 dsDNA with a 407-nt single-stranded gap containing a single-base change (C → T) in the template (plus) strand at position 75 of the *lacZα* coding sequence. This change created an ochre codon (TAA), resulting in a colorless plaque phenotype. The MF is defined as the proportion of blue plaques in the total number of plaques scored. Reactions were performed as described above for the forward mutation assays.

Coupled polymerase and exonuclease assays

The standard reaction (10 μl) contained 50 nM *Sso* pol B1, 2 nM ^{32}P-labeled substrate p30/t76 (prepared by annealing p30 to t76), 50 mM Tris–HCl or BisTris–Cl (adjusted to indicated pH), 2 mM β-mercaptoethanol, 100 μg/ml BSA, 3 mM $MgCl_2$, and 200 μM dNTPs. Reactions were carried out for 15 min at 65°C. The samples were analyzed by electrophoresis in a 17% polyacrylamide gel containing 8 M urea in 1× Tris–borate–EDTA. The gel was dried, exposed to X-ray film and analyzed by ImageQuant Storm PhosphorImager (Molecular Dynamics, Sunnyvale, USA).

3′–5′ Exonuclease assays

The standard reaction mixture (10 μl) contained an indicated amount of wild-type *Sso* pol B1 or D231A, 2 nM ^{32}P-labeled primer p42, 50 mM Tris–HCl, pH 8.0, 2 mM β-mercaptoethanol, 100 μg/ml BSA, and 3 mM $MgCl_2$. Reactions were carried out for 15 min at 65°C. The samples were subjected to eletrophoresis in a polyacrylamide-urea gel as described above. The gel was dried, exposed to X-ray film, and analyzed by ImageQuant Storm PhosphorImager (Molecular Dynamics, Sunnyvale, USA).

Steady-state kinetic assays

All steady-state kinetic reactions were performed at 55°C in 50 mM Tris–HCl, pH 6.5 (55°C), 2 mM β-mercaptoethanol, 0.1 mM EDTA, and 100 μg/ml BSA. *Sso* pol B1 exo$^-$ (5 nM) was pre-incubated with DNA substrate (250 nM) for 2 min at 55°C. Reactions were initiated by the addition of a preheated mixture of 4 mM $MgCl_2$ and increasing concentrations of a single nucleotide. Reactions were incubated for 2 min and quenched by the addition of 50 mM EDTA. The samples were subjected to eletrophoresis in a polyacrylamide-urea gel as described above. The gel was dried, exposed to X-ray film, and analyzed by ImageQuant Storm PhosphorImager (Molecular Dynamics, Sunnyvale, USA). Steady-state parameter K_m and V_{max} values for nucleotide incorporation were obtained by plotting the observed reaction rate (V_{obs}) values against the dNTP concentration and fitting the data to the Michaelis–Menten function. The incorporation efficiency of nucleotide was defined as the ratio of V_{max}/K_m. The frequency of misincorporation, f_{inc}, was measured by using the following equation: $f_{inc} = (V_{max}/K_m)_{incorrect}/(V_{max}/K_m)_{correct}$.

Results

Fidelity of *Sso* pol B1 in DNA synthesis at 70°C

M13-based assays, developed by Kunkel and his colleagues (Bebenek and Kunkel 1995), were employed to determine the fidelity of *Sso* pol B1. Three independent forward mutation experiments with wild-type *Sso* pol B1 were conducted at 70°C, yielding 335 mutant plaques in a total of 359,485 plaques. The *lacZα* MF of the wild-type enzyme was calculated to be 9.3×10^{-4} (Table 1). This number was slightly higher than the background MF (6×10^{-4}) (Bebenek and Kunkel 1995). In control experiments, the extraordinarily high-fidelity *Pfu* DNA polymerase (*Pfu* pol) (New England Biolabs, Ipswich, USA) was tested on the same batch of gapped substrates and under the same conditions as those used for *Sso* pol B1. The MF of *Pfu* pol was measured to be 5.6×10^{-4}, which was very close to the background value. Therefore, the difference between the MF of *Sso* pol B1 and the background resulted indeed from errors made by the enzyme in DNA synthesis. Although the fidelity of *Sso* pol B1 is slightly lower than that of *Pfu* pol, it is higher than that of DNA polymerase from *Thermococcus litoralis* (*Vent*™ pol) (Mattila et al. 1991), another thermostable high-fidelity DNA polymerase commercially available for PCR applications (Table 1). In general, it appears that known family B DNA polymerases from hyperthermophilic archaea are all capable of DNA synthesis with low error rates (Bae et al. 2009; Cline et al. 1996; Choi et al. 2008; Dietrich et al. 2002; Griffiths et al. 2007; Kim et al. 2007; Lee et al. 2009; Marsic et al. 2008; Mattila et al. 1991; Takagi et al. 1997). Furthermore, the forward MF of *Sso* pol B1 is similar to those of bacteriophage RB69 DNA polymerase (RB69 pol) (Bebenek et al. 2002) and T4 DNA polymerase (T4 pol) (Kroutil et al. 1998), and lower than those of several replicative DNA polymerases such as *Saccharomyces cerevisiae* DNA polymerase ε (*Sc* pol ε) (Shcherbakova et al. 2003) and *S. cerevisiae* DNA polymerase δ

Table 1 Mutant frequencies of *Sso* pol B1 and other DNA pols as determined in the M13-based forward mutation assays

DNA pol	dNTP (μM)	Temp (°C)	Plaques scored		MF[a] ($\times 10^{-4}$)	Exo^-/Exo^+	References
			Total	Mutant			
Sso pol B1							
Exo^+	1000	70	359,485	335	9.3	3.0	This study
Exo^-	1000	70	38,303	106	27.7		
Pfu pol	1000	72	32,414	18	5.6		This study
Vent™ pol							
Exo^+	1000	72	5,883	35	59	2.3	Mattila et al. (1991)
Exo^-	1000	72	4,438	59	133		
RB69 pol							
Exo^+	1000	37	54,411	52	9.6	3.1	Bebenek et al. (2002)
Exo^-	1000	37	91,799	276	30		
T4 pol							
Exo^+	100	37	–	–	9.2	6.1	Kroutil et al. (1998)
Exo^-	100	37	–	–	56		
Sc pol ε							
Exo^+	250	30	–	–	10	25	Shcherbakova et al. (2003)
Exo^-	250	30	–	–	250		
Sc pol δ							
Exo^+	250	30	–	–	44	5.2	Fortune et al. (2005)
Exo^-	250	30	–	–	227		
E. coli DNA polIII							
Exo^+	1000	30	126,465	468	37	3.2	Pham et al. (1998)
Exo^-	1000	30	78,005	924	118		

[a] The background MF (Bebenek and Kunkel 1995) is not subtracted

(*Sc* pol δ) (Fortune et al. 2005), and *E. coli* DNA polymerae III (*E. coli* DNA polIII) (Pham et al. 1998) (Table 1).

Spectrum of mutations generated by *Sso* pol B1

To determine the spectrum of mutations resulting from DNA synthesis by *Sso* pol B1, 79 independent mutant plaques obtained in the above forward mutation assays were picked, and the 407-bp region of M13mp2 DNA from each plaque was sequenced (Fig. 1). Like many other DNA polymerases (Bebenek et al. 2002; Cai et al. 1995; Kroutil et al. 1996), *Sso* pol B1 did not generate all types of errors at equal rates, but showed distinctive error specificity. Base substitution and frameshift were two major types of mutation (Table 2). Single-base substitutions were two-thirds of all errors, whereas frameshift mutations including single-base deletion (13) and insertion (3) accounted for 19% of the total mutants. The error rates of base substitution and frameshift for *Sso* pol B1 were calculated to be 8.1×10^{-6} and 1.5×10^{-6}, respectively (Table 2). Among the remainder of the mutations, repetition of a 7-nt (GAATTCA) sequence was found in two mutants and that of a 6-nt (TGCTTC) sequence in one mutant. These repetitive insertions have never been reported for other DNA polymerases. Interestingly, *Sso* pol B1 appeared to be more sequence-biased in generating errors than most of the other DNA polymerases and preferentially made mistakes only at a few 'hot spots' in the 407-nt region of the DNA substrate (e.g., +75C, −12T, +94A).

Substitution mutation by *Sso* pol B1

Since base substitution was a major type of errors made by *Sso* pol B1, we investigated it further by using an ochre codon reversion assay. The frequency of reversion mutation during gap filling by *Sso* pol B1 at 70°C was 8.3×10^{-6} (Table 3). This number is nearly identical to the single-base substitution frequency (8.1×10^{-6}) found in the forward mutation assays (Table 2). As a control, the MF of *Pfu* pol was measured to be 2.1×10^{-6} under the same assay conditions, which again is nearly identical to the background (2×10^{-6}) (Bebenek and Kunkel 1995). The reversion MF of *Sso* pol B1 is among the lowest in replicative DNA polymerases such as *Vent*™ pol, *Sc* pol ε, and *Sc* pol δ, and about two to four orders of magnitudes lower than polymerases participating in DNA transactions other than replication, e.g., as thermostable *Taq* DNA polymerase

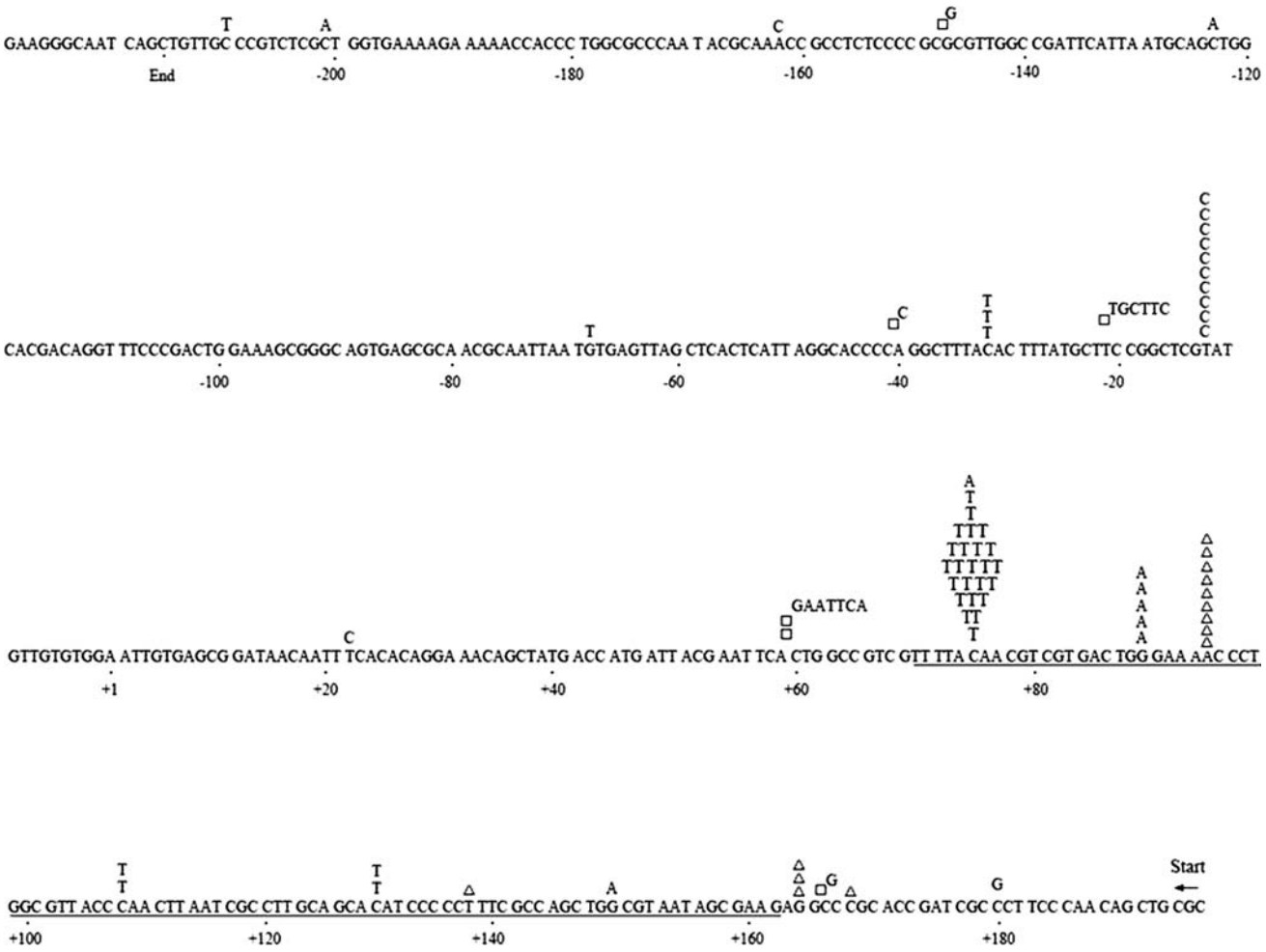

Fig. 1 Types of errors generated by *Sso* pol B1 during gap-filling DNA synthesis in the M13-based forward mutation assays. Single-base substitutions are shown above the template sequence. Base additions are shown with a "open square" at positions where addition(s) occurred, and the added base(s) are indicated. Single-base deletions are shown by a "open triangle." A 93-base deletion is underlined

(*Taq* pol) (Tindall and Kunkel 1988) and human DNA polymerase η (human pol η) (Matsuda et al. 2000) (Table 3).

Contribution of exonucleolytic proofreading to the fidelity of *Sso* pol B1

We have previously shown that *Sso* pol B1 possesses a strong 3′–5′ exonuclease activity (Lou et al. 2004b). To determine the contribution of the exonuclease activity to the fidelity of *Sso* pol B1, an exonuclease-deficient mutant of *Sso* pol B1 (D231A) was constructed by substituting an alanine residue for the conserved residue D231 located in motif *Exo*I. As shown in an excision assay (Fig. 2), an exonuclease-deficient mutant of *Sso* pol B1 (D231A) was only ~5% as active as the wild-type enzyme in exonuclease activity while this mutant protein remained fully active in DNA synthesis. In the M13-based assays, the forward MF in gap filling by the mutant protein (27.7×10^{-4}) was approximately three times as high as that observed with wild-type *Sso* pol B1. Similarly, the frequency of reversion of the ochre codon by D231A (24.3×10^{-6}) was also three times as high as that by wide-type *Sso* pol B1. The ratios of the mutant frequency of mutant *Sso* pol B1 to that of the wild-type protein (Exo^-/Exo^+ ratios) resembled those of *Vent*^{TM} pol (Mattila et al. 1991), RB69 pol (Bebenek et al. 2002), and *E. coli* DNA polIII (Pham et al. 1998) but lower than those of *Sc* pol ε (Shcherbakova et al. 2003), *Sc* pol δ (Fortune et al. 2005), and T4 pol (Kroutil et al. 1998) (Tables 1 and 3), suggesting that accurate polymerization, rather than proofreading, contributes most significantly to the high fidelity of the enzyme.

Effects of temperature, pH, metal cofactors, and Sul7d on the fidelity of *Sso* pol B1

Since *Sulfolobus* thrives in an acidic and hot environment, it is of particular interest to understand how the organism and

Table 2 Base substitutions and frameshifts generated by *Sso* pol B1 in the M13-based forward mutation assays

Error types	No.	Mutant fractions (%)	MF[a] ($\times 10^{-4}$)	Error rate ($\times 10^{-6}$)
C·dATP				
C → T	32	38.1	3.5	23.6
C·dCTP				
C → G	1	1.2	0.1	≤2.1
C·dTTP				
C → A	3	3.6	0.3	3.5
A·dCTP				
A → G	0	<1.2	<0.1	<1.0
A·dGTP				
A → C	1	1.2	0.1	≤1.1
A·dATP				
A → T	0	<1.2	<0.1	<1.0
T·dCTP				
T → G	0	<1.2	<0.1	<1.0
T·dTTP				
T → A	0	<1.2	<0.1	<1.2
T·dGTP				
T → C	11	13.1	1.2	7.5
G·dGTP				
G → C	0	<1.2	<0.1	<1.0
G·dATP				
G → T	1	1.2	0.1	≤0.7
G·dTTP				
G → A	6	7.1	0.7	5.0
Total single base substitution	55	65.5	6.0	8.1
−1 deletion	13	15.5	1.4	1.2
+1 addition	3	3.6	0.3	0.3
Total single base frameshift	16	19.1	1.7	1.5
Others	13	15.5		
Total	84		9.3×10^{-4}	

[a] The background MF (Bebenek and Kunkel 1995) is not subtracted

its fundamental biological processes such as DNA replication adapt to the extreme conditions. In this study, we measured the reversion MF of *Sso* pol B1 under various gap-filling reaction conditions (Table 4). When the reaction temperature was lowered from 70 to 55°C, the reversion MF of *Sso* pol B1 increased by 2.9-fold to 23.7×10^{-6}. This is similar to the finding that the MF of *Vent*^{TM} pol decreased in the forward mutation assay when reaction temperature was raised from 55 to 72°C (Mattila et al. 1991). When the pH of the reaction buffer was adjusted from 6.5 to 8.0, the reversion MF of *Sso* pol B1 increased by 2.6-fold to 21.5×10^{-6}. In a coupled polymerase-exonuclease assay, pH showed clear opposing effects on the 5′–3′ polymerase and 3′–5′ exonuclease activities of the enzyme (Fig. 3). While DNA polymerization occurred optimally at pH 5–7, exonucleolytic degradation became more pronounced with increasing alkalinity. Therefore, the increased fidelity of *Sso* pol B1 at lower pH presumably resulted more from accurate polymerization than from proofreading. The polymerase may undergo conformational changes favorable for template binding and base selection at a slightly acidic pH (Eckert and Kunkel 1993). *Sso* pol B1 is active in the presence of either Mg^{2+} or Mn^{2+}, with the former being the preferred cofactor for polymerization activity and the latter for exonuclease activity (data not shown). The reversion MF of *Sso* pol B1 was enhanced by 2.1-fold to 17.2×10^{-6} when Mn^{2+} instead of Mg^{2+} was used in the gap-filling reactions. Similar effects of substituting Mn^{2+} for Mg^{2+} on the fidelity of DNA synthesis were also observed with Dpo4 (Vaisman et al. 2005) and DNA polymerase β (Werneburg et al. 1996).

Genomic DNA is coated with Sul7d, a group of 7-kDa chromatin proteins, in *Sulfolobus* (Mai et al. 1998). We have previously shown that Ssh7, a Sul7d protein from *Sulfolobus shibatae*, is able to stimulate the polymerization and inhibit the exonuclease activities of *Sso* pol B1 without impairing the proofreading function of the enzyme in vitro (Lou et al. 2004a). In agreement with this observation, we found that reversion of the ochre codon mutation by *Sso* pol B1 in the presence and absence of Ssh7 also occurred with similar frequencies (8.1×10^{-6} vs. 8.3×10^{-6}). Therefore, the major *Sulfolobus* chromatin protein does not affect the fidelity of *Sso* pol B1 in DNA synthesis.

Kinetic analysis of the polymerization fidelity of *Sso* pol B1

The M13-based mutational assays suggest that the low MF of gap filling by *Sso* pol B1 is attributable to the high polymerization fidelity of the enzyme. To learn more about the fidelity of DNA polymerization by *Sso* pol B1, we determined the nucleotide incorporation kinetics of an exonuclease-deficient triple-point mutant of the enzyme (*Sso* pol exo$^-$) exo$^-$ under steady-state conditions. To permit easy quantitation of the products resolved by gel electrophoresis while preventing the substrate from denaturation, the assays were carried out at 55°C on a primed template containing a short primer ($T_m = \sim 63$°C). In a representative experiment, where incorporation of correct and incorrect nucleotides opposite the template base G residue was measured, *Sso* pol B1 exo$^-$ was incubated with an excess amount of the DNA substrate, and the extension products were resolved by gel electrophoresis and quantified (Fig. 4a). Data were fit by nonlinear regression to yield the kinetic curves for correct and incorrect incorporations (Fig. 4b, c). Steady-state kinetic parameters (K_m and V_{max})

Table 3 Mutant frequencies of *Sso* pol B1 and other DNA pols as determined in the M13-based reversion mutation assays

DNA pol	dNTP (μM)	Temp (°C)	Plaques scored		MF[a] ($\times 10^{-6}$)	Exo$^-$/Exo$^+$	References
			Total	Mutant			
Sso pol B1							
Exo$^+$	1000	70	844,000	7	8.3	2.9	This study
Exo$^-$	1000	70	452,000	11	24.3		
Pfu pol	1000	72	1,442,400	3	2.1		This study
Vent^{TM} pol							
Exo$^+$	1000	72	795,000	48	60	3.2	Mattila et al. (1991)
Exo$^-$	1000	72	280,000	54	193		
T4 pol							
Exo$^+$	100	37	–	–	2.5	7.2	Kroutil et al. (1998)
Exo$^-$	100	37	–	–	18		
Sc pol ε							
Exo$^+$	250	30	–	–	33	23.6	Shcherbakova et al. (2003)
Exo$^-$	250	30	–	–	780		
Sc pol δ							
Exo$^+$	250	30	–	–	23	60.9	Fortune et al. (2005)
Exo$^-$	250	30	–	–	1,400		
Taq pol	1000	70	590,000	184	312		Tindall and Kunkel (1988)
Human pol η	500	37	–	–	42,000		Matsuda et al. (2000)

[a] The background MF (Bebenek and Kunkel 1995) is not subtracted

Fig. 2 Analysis of the 3′–5′ exonuclease activity of wild-type and exonuclease-deficient mutant of *Sso* pol B1. Wild-type or exonuclease-deficient mutant of *Sso* pol B1 (D231A) was incubated with oligonucleotide p42 (2 nM) at 65°C for 15 min. Samples were resolved by denaturing electrophoresis in a urea-polyacrylamide gel. The gel was dried and exposed to X-ray film. *Lane C* no enzyme, *lane 1* 50 nM exonuclease-deficient mutant of *Sso* pol B1 (D231A), *lane 2* 100 nM exonuclease-deficient mutant of *Sso* pol B1(D231A), *lane 3* 20 nM wild-type *Sso* pol B1

for each nucleotide were obtained by fitting these data to the Michaelis–Menten equation (Table 5). For correct dCTP incorporation, the K_m and V_{max} values were 0.048 ± 0.009 μM and 41.7 ± 0.6 nM/min, respectively. The K_m of *Sso* pol B1 exo$^-$ is similar to that of exonuclease-deficient *Pfu* pol (Rudinger et al. 2006), but is about two orders of magnitudes lower than those of DNA polymerases from *Thermococcus* species (Kong et al. 1993; Marsic et al. 2008; Southworth et al. 1996) for incorporating a correct dNTP. For incorrect dATP incorporation, the K_m and V_{max} values of *Sso* pol B1 exo$^-$ were 157 ± 15 μM and 12.6 ± 0.2 nM/min, respectively. Thus, the frequency of misincorporating dATP, f_{inc}, was calculated to be 9.2×10^{-5}. Similarly, the f_{inc}'s of misincorporating dGTP and dTTP were 8.2×10^{-5} and 5.4×10^{-5}, respectively (Table 5). The frequencies of all possible misincorporations varied within a range from 5.4×10^{-5} for inserting a T opposite the template base G to 4.6×10^{-4} for inserting a G opposite the template base T with an estimated f_{inc} average of 1.8×10^{-4}, suggesting that *Sso* pol B1 exo$^-$ introduced one error for every 10^4–10^5 nucleotide incorporated in vitro at 55°C (Table 5).

Discussion

The fidelity of DNA polymerases varies widely in relation to their biological functions and origins (Kool 2002). Replicative DNA polymerases are known to exhibit high fidelity, which is essential for maintaining genome stability

Table 4 Mutant frequencies of *Sso* pol B1 under various conditions as determined in the reversion mutation assays

Temp (°C)	pH	Metal ions	Ssh7 (25 μM)	Plaques scored		MF[a] ($\times 10^{-6}$)
				Total	Mutant	
55	6.5	Mg^{2+}	–	506,000	12	23.7
70	6.5	Mg^{2+}	–	844,000	7	8.3
70	8.0	Mg^{2+}	–	790,000	17	21.5
70	6.5	Mn^{2+}	–	990,000	17	17.2
70	6.5	Mg^{2+}	+	1,110,000	9	8.1

[a] The background MF (Bebenek and Kunkel 1995) is not subtracted

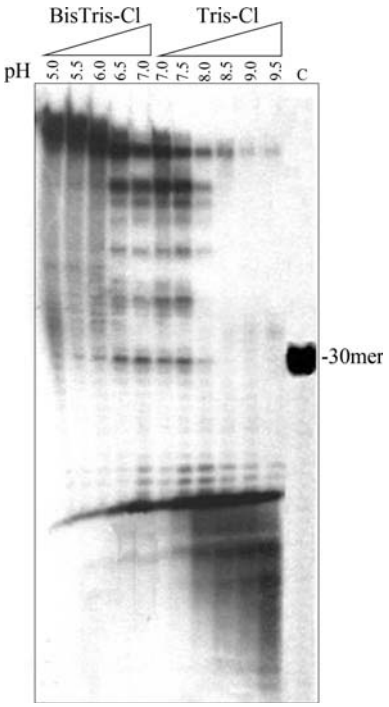

Fig. 3 Effect of pH on the 5′–3′ polymerase and 3′–5′ exonuclease activities of *Sso* pol B1. *Sso* pol B1 (75 nM) was incubated with substrate p30/t76 (2 nM) for 15 min at 65°C in the presence of 200 μM dNTPs at various pH values. Samples were resolved by denaturing electrophoresis in a urea-polyacrylamide gel. The gel was dried and exposed to X-ray film

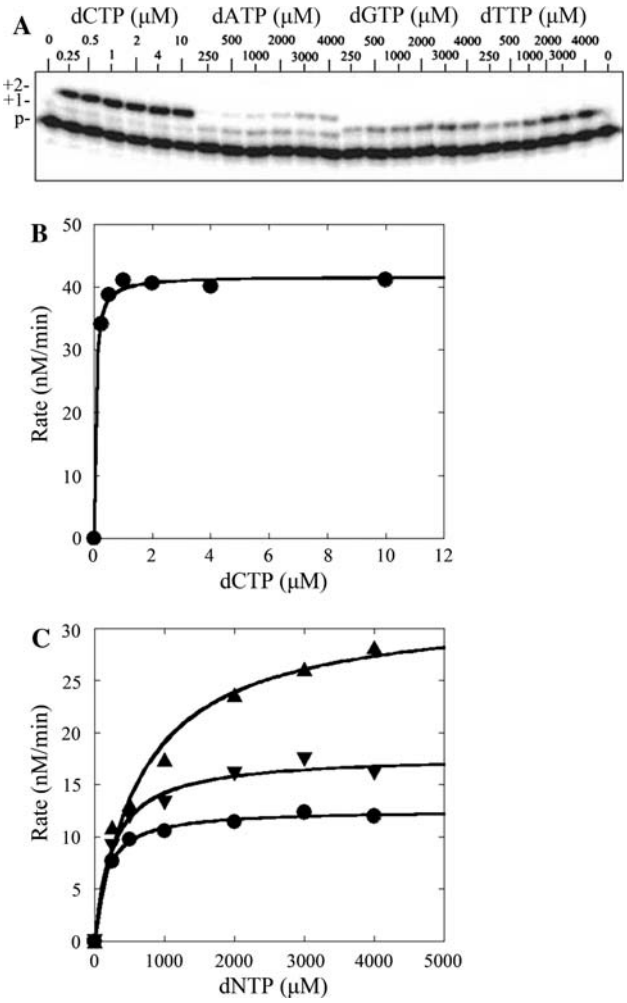

Fig. 4 Nucleotide incorporations by *Sso* pol B1 exo$^-$ opposite the template G residue. **a** A gel image for dCTP, dATP, dGTP, and dTTP incorporations. Reactions were performed and terminated as described in the section "Material and methods." Samples were resolved by denaturing electrophoresis in a urea-polyacrylamide gel. Positions of the primer (*p*) and the extension products (+1 and +2) are indicated. **b** Quantitation of correct dCTP incorporation. Observed rates for correct dCTP incorporation were determined and plotted against dCTP concentrations. The V_{max} and K_m values obtained by fitting the data to the Michaelis–Menten function are listed in Table 5. **c** Quantitation of dATP, dGTP, and dTTP misincorporations. Observed rates for dATP (*filed circle*), dGTP (*filled inverse triangle*), and dTTP (*filled triangle*) incorporations were determined and plotted against dATP, dGTP, and dTTP concentrations, respectively. The V_{max} and K_m values obtained by fitting the data to the Michaelis–Menten function are listed in Table 5

in cells. To understand the potential function of *Sso* pol B1 in the hyperthermophilic archaeon *S. solfataricus*, we investigated the fidelity of the enzyme by using M13-based genetic assays at near the physiological temperature for the first time. The MF of *Sso* pol B1, as determined by both forward and reversion mutation assays, is typical of that of a replicase since it is close to those of bacterial or viral replicases, such as *E. coli* DNA polIII holoenzyme (Pham et al. 1998), RB69 pol (Bebenek et al. 2002), and T4 pol (Kroutil et al. 1998), and lower than most eukaryotic replicases, such as *Sc* pol ε (Shcherbakova et al. 2003) and *Sc* pol δ (Fortune et al. 2005). These results agree with the notion that *Sso* pol B1 serves as a replicative DNA polymerase.

The rate of spontaneous mutation in *S. acidocaldarius* was measured to be $\sim 7.8 \times 10^{-10}$ per base (Grogan et al. 2001). Estimates of rates of spontaneous mutation in *S. solfataricus* are at least 50–60 folds higher than that for *S. acidocaldarius* (Martusewitsch et al. 2000). It appears that the error rate of *Sso* pol B1, as determined by the M13-

Table 5 Kinetic parameters for nucleotide incorporation by *Sso* pol B1 exo$^-$ at 55°C

dNTP	V_{max} (nM/min)	K_m (μM)	V_{max}/K_m	f_{inc}^a
Template A				
dTTP	18.4 ± 0.1	0.032 ± 0.003	5.8×10^2	
dATP	20.6 ± 0.8	492 ± 81	4.2×10^{-2}	7.2×10^{-5}
dCTP	9.4 ± 0.3	109 ± 33	8.6×10^{-2}	1.5×10^{-4}
dGTP	15.7 ± 0.1	143 ± 4	1.1×10^{-1}	1.9×10^{-4}
Template T				
dATP	23.3 ± 0.8	0.099 ± 0.025	2.4×10^2	
dCTP	20.9 ± 0.7	345 ± 53	6.1×10^{-2}	2.5×10^{-4}
dGTP	25.5 ± 0.6	239 ± 30	1.1×10^{-1}	4.6×10^{-4}
dTTP	28 ± 1	391 ± 61	7.2×10^{-2}	3.0×10^{-4}
Template G				
dCTP	41.7 ± 0.6	0.048 ± 0.009	8.7×10^2	
dATP	12.6 ± 0.2	157 ± 15	8.0×10^{-2}	9.2×10^{-5}
dGTP	17.8 ± 0.6	251 ± 40	7.1×10^{-2}	8.2×10^{-5}
dTTP	32 ± 2	674 ± 120	4.7×10^{-2}	5.4×10^{-5}
Template C				
dGTP	19.5 ± 0.2	0.05 ± 0.01	3.9×10^2	
dATP	19.6 ± 0.8	258 ± 49	7.6×10^{-2}	1.9×10^{-4}
dCTP	14.8 ± 0.4	152 ± 27	9.7×10^{-2}	2.5×10^{-4}
dTTP	22.2 ± 0.7	484 ± 63	4.6×10^{-2}	1.2×10^{-4}

[a] $f_{inc} = (V_{max}/K_m)_{incorrect}/(V_{max}/K_m)_{correct}$

based assays, is about two orders of magnitude higher than the in vivo rate of spontaneous mutation in *S. solfataricus*. Conceivably, other mechanisms, such as DNA repair, in addition to accurate DNA synthesis by *Sso* pol B1, are required to ensure the high fidelity of genomic DNA replication in *S. solfataricus*.

Base substitution is the major type of errors created by *Sso* pol B1, accounting for about two-thirds of total mutants produced in the gap-filling reaction. It is worth noting that 58% of the total substitution mutants produced by *Sso* pol B1 involved a transition from C to T. This is the first report of the in vitro mutation spectrum of a replicative DNA polymerase from *Sulfolobus*. Similar rates of base substitution were observed in *E. coli* DNA polymerase II (61%) (Cai et al. 1995), T7 DNA polymerase (55%) (Kroutil et al. 1996), and RB69 pol (59%) (Bebenek et al. 2002). The C → T transition often occurs following deamination of a cytosine to form a uracil in DNA and the subsequent change of the G:C pair into the A:T pair via a G:U intermediate (De Felice et al. 2007). The rate of hydrolytic deamination at 60°C was about 20-fold higher than that at 37°C (Frederico et al. 1993). Recently, *Sso* pol B1 has been shown to be capable of sensing uracil in the template strand and pausing 3–4 bp upstream the lesions by the 'read ahead' mechanism (Savino et al. 2004). However, extension beyond the lesions by the enzyme was not as effectively blocked as in the case of *Pfu* pol (Gruz et al. 2003). The ability of *Sso* pol B1 to introduce the C → T transition mutation is consistent with this finding.

Spectra of spontaneous mutation have been investigated in several *Sulfolobus* species (Berkner and Lipps 2007, 2008; Grogan et al. 2001; Redder and Garrett 2006). The spontaneous mutation in *S. acidocaldarius* included base substitutions and insertions and deletions of various sizes, but the proportion of base substitutions was low (12%) (Grogan et al. 2001). *S. solfataricus* P1 and P2 appeared to have a higher spontaneous mutation frequency and a higher proportion of insertion mutation than *S. acidocaldarius* (Grogan et al. 2001; Martusewitsch et al. 2000; Redder and Garrett 2006). Since the spectrum of spontaneous mutation is determined by many factors including errors in DNA replication, mismatch correction by proofreading, post-replication DNA mismatch repair, DNA damage leading to mutations during inaccurate repair, and unrepaired DNA damage, it is not surprising that the spectrum of errors produced by *Sso* pol B1 differs from those of spontaneous mutation in various *Sulfolobus* species. However, it is worth noting that, as found in the error spectrum of *Sso* pol B1, most base substitutions were transitions in *S. acidocaldarius* and *S. solfataricus* P2 (Grogan et al. 2001; Redder and Garrett 2006). In comparison, spontaneous point mutations introduced by *Pfu* pol were predominantly transversions (André et al. 1997).

Most polymerases responsible for genome replication contain an intrinsic 3′–5′ exonucleolytic proofreading function. The exonuclease-deficient mutant of *Sso* pol B1 created three times as many mutants as the wild-type enzyme in both forward and reversion mutation assays. Similarly, the mutant frequencies of *Vent*^{TM} pol in forward and reversion mutation assays were two to four folds lower than the respective values obtained with the enzyme deficient in proofreading (Mattila et al. 1991). In contrast, the error rates of the exonuclease-deficient mutants of *Pfu* pol and *Thermococcus zilligii* DNA polymerase (*T. zilligii* pol) are ~40- and ~30-fold higher than those of the exonuclease-proficient forms of the two polymerases, respectively (Cline et al. 1996; Griffiths et al. 2007). The relatively low Exo$^-$/Exo$^+$ ratio of MF of *Sso* pol B1, as compared to those of the majority of DNA polymerases capable of proofreading, raised the possibility that the high fidelity of *Sso* pol B1 is primarily contributed by accuracy in nucleotide incorporation.

Kinetic analysis of nucleotide incorporation by *Sso* pol B1 exo$^-$ showed that the K_m values for correct nucleotide incorporations were three to four orders of magnitude lower than those for nucleotide misincorporations (Table 5), suggesting that an incorrect nucleotide in the active site of the enzyme had a much greater tendency to dissociate than a correct nucleotide. This is consistent with the finding that

the binding affinity of *Sso* pol B1 exo$^-$ for a correct nucleotide was about 1000-fold higher than that for an incorrect nucleotide (Zhang et al. 2009). On the other hand, the V_{max} values for correct and incorrect incorporations were much closer (Table 5). In comparison, large differences in K_m were observed in steady-state kinetic analyses of several DNA polymerases, such as *Drosophila* DNA polymerase α (Petruska et al. 1988) and *E. coli* DNA polIII (Sloane et al. 1988), whereas a strict V_{max} discrimination against incorrect dNTPs was found with *E. coli* DNA polI (Kuchta et al. 1988) and herpes simplex virus type 1 DNA polymerase exonuclease-deficient mutant (Song et al. 2004). Taken together, our results indicate that the high fidelity of *Sso* pol B1 exo$^-$ in DNA synthesis is contributed primarily by K_m rather than V_{max} difference.

In conclusion, we have shown that *Sso* pol B1 is capable of high-fidelity DNA synthesis at near the optimal growth temperature for *S. solfataricus*. Errors made by the enzyme in DNA synthesis are predominated by single-base substitutions and deletions. The low MF of *Sso* pol B1 is attributable to the high polymerization fidelity of the enzyme, which is based primarily on K_m discrimination against incorporating incorrect nucleotides. Our results are consistent with the suggestion that *Sso* pol B1 serves as a replicative DNA polymerase in *S. solfataricus*.

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