Kinetic study of various binding modes between human DNA polymerase β and different DNA substrates by surface-plasmon-resonance biosensor

Pui Yan TSOI and Mengsu YANG¹

Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong, People's Republic of China

The interaction of a series of DNA substrates with human DNA polymerase β has been studied in real time by using a surface-plasmon-resonance (SPR) biosensor technique. We have prepared the sensor surfaces comprising different DNA targets, including single-stranded DNA, blunt-end double-stranded DNA, gapped DNA and DNA template–primer duplexes containing various mismatches at different positions. The binding and dissociation of polymerase β at the DNA-modified surfaces was measured in real time, and the kinetics profiles of polymerase–DNA interaction were analysed using various physical models. The results showed that polymerase β binding to single-stranded DNA ($K_A = 1.25 \times 10^8 \text{ M}^{-1}$; where K_A is the equilibrium affinity constant) was thermodynamically more favourable than to blunt-end DNA duplex ($K_A = 7.56 \times 10^7 \text{ M}^{-1}$) or gapped DNA ($K_A = 8.53 \times 10^7 \text{ M}^{-1}$), with a single binding mode on each DNA substrate. However, polymerase β bound to DNA template–primer duplexes (15 bp with a 35 nt overhang) at two

sites, presumably one at the single-strand overhang and the other at the 3'-end of the primer. When the DNA duplex was fully matched, most of the polymerase β (83%) bound to the template–primer duplex region. The introduction of different numbers of mismatches near the 3'-end of the primer caused the binding affinity and the fraction of polymerase β bound at the duplex region to decrease 8–58-fold and 15–40%, respectively. On the other hand, the affinity of polymerase β for the single-strand overhang remained unchanged while the fraction bound to the single-strand region increased by 15–40%. The destabilizing effect of the mismatches was due to both a decrease in the rate of binding and an increase in the rate of dissociation for polymerase β .

Key words: human DNA polymerase β , DNA primer–template duplex, molecular recognition, parallel binding, SPR biosensor.

INTRODUCTION

DNA polymerases play a central role in several DNA transactions *in vivo*, including DNA replication, recombination and base-excision DNA repair [1,1a]. DNA replication involves binding of polymerase to the DNA template–primer duplex and subsequent incorporation of a dNMP substrate into a growing DNA polymer. DNA polymerases discriminate between correct and incorrect dNTPs mainly during binding of the substrate by an induced-fit mechanism [2]. The incorporation of errors into the genome occurs when a polymerase fails to discriminate between the correct and incorrect dNTP substrates, resulting in genetic aberrations or genomic instability. Therefore, it is important to understand the molecular mechanisms that DNA polymerases employ to recognize DNA mismatches.

DNA polymerase β (pol β), the smallest of the four nuclear DNA polymerases (α , β , δ and ϵ) [1,1a,3], plays an important role in gap-filling synthesis during base-excision repair in mammals [3–9]. In contrast to other DNA polymerases, catalysis by pol β fits a distributive model that requires the enzyme to dissociate from and rebind to the template during each dNMP incorporation cycle [3,10,11]. Pol β is present in all mammalian tissues and is generally expressed at low levels, similar to a number of other so-called constitutive 'housekeeping' enzymes [3,12]. Human pol β is a single-chain polypeptide of 335 amino acids [13–15], consisting of a 31 kDa C-terminal domain that includes the polymerase active site [16,17] and an 8 kDa N-terminal domain that participates in binding to DNA and exhibits 5'-3' deoxyribose phosphodiesterase (lyase) activity [18]. The lack of intrinsic exonuclease activity in pol β [6,19] results in its error

rates being higher than those of the other cellular polymerases [20–22].

High-resolution structures of rat and human pol β [16,23–25], together with site-directed mutagenesis experiments [26] and biophysical studies [17,27,28], have provided us with comprehensive knowledge of the structure–function relationship of the DNA–pol β complex. The 8 kDa domain of ≈ 80 amino acids has a significant degree of α -helical structure, whereas the 31 kDa fragment of ≈ 250 amino acids possesses dominant β -sheet structure [27,29,30]. Both single- and double-stranded nucleic acids bind with intact pol β . The 8 kDa domain has a high affinity for single-stranded DNA (ssDNA), whereas the C-terminal 31 kDa domain binds double-stranded DNA (dsDNA) weakly and has catalytic activity [17,27,28]. Although some studies have shown that the 31 kDa domain has no ssDNA-binding activity [17,27,28], early fluorescence studies indicated that human pol β forms a complex with a ssDNA in which 16 nt are occluded by the enzyme; this complex was designated (pol β)₁₆ [31]. In the (pol β)₁₆-binding mode, the 31 kDa catalytic domain of the enzyme was also involved in interactions with the ssDNA. It has also been shown that pol β has affinity for duplex DNA, containing nicks bearing the 3' ends of upstream primers and the 5' termini of downstream primers [7,8,32,33], and that pol β prefers to bind to both the template and primer strands.

The surface-plasmon-resonance (SPR) biosensor technique is a useful tool for obtaining quantitative kinetic and affinity information on biomolecular interactions [34–36]. An SPR biosensor can translate a biospecific interaction between a ligand in solution and a binding partner immobilized on the surface into a detectable signal that is directly proportional to the extent of

Abbreviations used: SPR, surface plasmon resonance; pol β , polymerase β ; SA5, streptavidin-modified; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; RU, resonance units.

¹ To whom correspondence should be addressed (e-mail bhmyang@cityu.edu.hk).

the interaction. Binding-affinity studies using an SPR biosensor have contributed significantly to understanding of the molecular basis of DNA–protein interactions [37–39]. In this article, we describe a study of the interactions between pol β and different DNA substrates using an SPR biosensor. Unlike other cellular DNA polymerases, pol β lacks 3′–5′ exonuclease activity [6,20], making it a simple and convenient target for mechanistic studies of DNA binding. Although the structures of complexes and modes of interaction between pol β and selected DNA have been investigated by other techniques [7,8,17,24,27,28,31], and the rate of polymerization and the apparent equilibrium dissociation constant of dNTP have been determined by pre-steady-state kinetic analysis [40], the binding kinetics and affinity of DNA–pol β interactions in the absence of polymerization have not been determined. In this report, a detailed kinetic study of the various proposed modes of binding between pol β and different DNA targets was carried out. The binding characteristics of pol β to various DNA substrates, including ssDNA, dsDNA, template–primer duplexes, and nicked and gapped DNA were determined and compared systematically to establish the binding preference of pol β . Furthermore, the effects of mismatch(es) at the 3′-end of the primer in different template–primer duplexes on pol β -binding affinity and binding modes were investigated to estimate the sensitivity of pol β to mispaired DNA. This is the key to understanding how the enzyme discriminates between correct and incorrect bases.

MATERIALS AND METHODS

Materials and equipment

The SPR biosensor instrument (BIAcore X) and sensor chips [streptavidin-modified (SA5) research grade] were acquired from Pharmacia Biosensor (Uppsala, Sweden). Human DNA pol β was purchased from Chimex (Milwaukee, WI, U.S.A.). All the experiments with pol β were carried out in TSB buffer (50 mM Tris/HCl, pH 7.4/100 mM NaCl). Individual DNA pol β (20–100 nM) in TSB was used for the DNA–polymerase binding experiments. NaCl was purchased from Acros (Geel, Belgium) and 1 M NaCl was prepared in order to regenerate the DNA-modified surface by removing protein from DNA substrates. Tris, Hepes and MgCl₂ were acquired from Sigma (St Louis, MO, U.S.A.). HSM buffer (10 mM Hepes, pH 7.4/150 mM NaCl/10 mM MgCl₂) and TSB buffer (pH 7.4) were used as the DNA immobilization buffer and running buffer, respectively, in all the experiments.

Design of DNA substrates

One 5′-biotin-labelled 50-mer single-stranded oligonucleotide (GCTEM-5B template, Figure 1A), six unlabelled 15-mer oligonucleotides (upstream primers), one unlabelled 50-mer oligonucleotide (complementary strand) and one unlabelled 27-mer oligonucleotide (downstream primer) were synthesized and purified by HPLC (DNAgency, Malvern, PA, U.S.A. and Invitrogen Life Technologies, Carlsbad, CA, U.S.A.). The sequences of the oligonucleotides are shown in Figure 1. To facilitate annealing of the oligonucleotides to form duplexes, equimolar amounts of oligonucleotides were mixed together in HSM buffer. The mixtures were incubated at 100 °C for 5 min and allowed to cool slowly to room temperature. The complementary oligonucleotide (50-mer) formed fully matched blunt-end DNA with GCTEM-5B template (Figure 1B). The primers GCPM-1 and GCPM-R annealed to the template to form a gapped DNA duplex with an 8-mer gap in the middle (Figure 1C). For DNA template–primer duplexes, the primer GCPM-1 formed a fully matched DNA

A

Single-stranded DNA template

3′-CGTCGGCAGGTTCCC GTCAGAACCATAGTCAGCGATTGCACGCAACTACG -5′B

B

Blunt-end DNA duplex

3′-CGTCGGCAGGTTCCC GTCAGAACCATAGTCAGCGATTGCACGCAACTACG -5′B
5′-GCAGCCGTCCAAGGG CAGTCT TGGTATCAGTCGCTAACGTGCGTTGATGC -3′

C

Gapped DNA duplex

3′-CGTCGGCAGGTTCCC GTCAGAACCATAGTCAGCGATTGCACGCAACTACG -5′B
5′-GCAGCCGTCCAAGGG GTATCAGTCGCTAACGTGCGTTGATGC -3′
(GCPM-1) (GCPM-R)

D

DNA template–primer duplex

3′-CGTCGGCAGGTTCCC GTCAGAACCATAGTCAGCGATTGCACGCAACTACG -5′B
5′-GCAGCCGTCCAAGGG (GCPM-1)
5′-GCAGCCGTCCAAGGT (GCPM-2)
5′-GCAGCCGTCCAAGTG (GCPM-3)
5′-GCAGCCGTCCAATGG (GCPM-4)
5′-GCAGCCGTCCAAGTT (GCPM-5)
5′-GCAGCCGTCCAATT (GCPM-6)

Figure 1 DNA substrates used for pol β binding

(A) Single-stranded 5′-biotin-labelled DNA (GCTEM-5B, 50-mer), also known as the template. (B) A complementary oligonucleotide (50-mer) is annealed with the template to form a blunt-end fully matched DNA duplex. (C) The upstream primer, GCPM-1, and the downstream primer, GCPM-R, are annealed with the DNA template to form a gapped DNA duplex, with a gap of 8 nt. (D) The primer GCPM-1 (15-mer) is fully complementary to the template strand. The other primers (GCPM-2–6, 15-mer) contain mismatched bases at different positions and form DNA duplexes with the template.

duplex with the GCTEM-5B template, whereas the other primers (GCPM-2–6) formed duplexes with the template containing various numbers of mismatched bases at different positions (see Figure 1D).

Biosensor measurement of pol β –DNA interaction

The SA5 sensor surface was first equilibrated with HSM buffer before DNA immobilization. When a stable baseline was observed, a 35 μ l solution of DNA substrate (2 μ M in HSM buffer) was injected on to the sensor chip for 7 min. After the injection, the DNA-modified surface was washed with HSM buffer to remove the unbound biotin-labelled DNA molecules until a stable baseline was obtained. The immobilization procedure was carried out at 25 °C and a constant flow rate of 5 μ l/min. To correct for non-specific binding and bulk refractive-index change, a blank channel containing buffer only was used as the control. The amount of ligand immobilized was calculated using the difference in the response levels before and after ligand immobilization, where the sensor response [1000 resonance units (RU)] was estimated to equal a surface concentration of ≈ 1 ng $\cdot$ mm^{−2} [41]. All binding experiments were carried out at 25 °C with a constant flow rate of 5 μ l/min in TSB buffer. A 15 μ l solution of pol β at different concentrations (20–100 nM) in TSB buffer was injected over the DNA-modified sensor surface for 3 min, followed by washing with TSB buffer for 5 min. The DNA-modified surfaces were regenerated by injecting 10 μ l of 1 M NaCl for 2 min. The sensor surface without a DNA coating was used as the control and was run simultaneously for each binding experiment. The

sensorgrams for all DNA–polymerase-binding interactions were recorded in real time and were analysed after subtracting the sensorgram from the controlled channel.

Data analysis

The binding and dissociation data were modelled and analysed by using BIAevaluation software, version 3.0 (Pharmacia Biosensor AB, Uppsala, Sweden). We used a simple 1:1 (Langmuir) model and a heterogeneous ligand 1:2 (parallel binding) model to fit the data by using the numerical integration method. A mass-transfer rate constant (k_{mt}) was included in the models to account for diffusion of molecules of analyte (pol β) from the solution to the biosensor interface according to Fick's law [42,42a,43]. The data were usually analysed by fitting both the association and dissociation phases for several concentrations. The degree of randomness of the residual plot and the reduced χ^2 value were used to assess the appropriateness of the various models for analysing the sensor data.

RESULTS AND DISCUSSION

Pol β –DNA binding modes

Based on previous structural and biophysical studies [16–33], models of binding of pol β to various DNA substrates are shown in Figure 2. It is assumed that, at low concentrations [31] (the

concentration range of 20–100 nM used in this study), pol β binds to ssDNA, dsDNA and gapped DNA (with the gap smaller than 16 nt), with 1:1 binding.

The binding mechanism for the interaction between pol β and ssDNA involves the 8 and 31 kDa domains both binding to ssDNA, occupying 16 nt (Figure 2A). The kinetics describing this 1:1 (Langmuir) model can be written as:

$$d[A]/dt = k_{mt}([A]_{\text{solution}} - [A]) - (k_a[A][B] - k_d[AB]) \quad (1)$$

$$d[B]/dt = -(k_a[A][B] - k_d[AB]) \quad (2)$$

where A and B represent pol β in solution and DNA immobilized on the sensor surface, respectively, k_a is the association rate constant, k_d is the dissociation rate constant and k_{mt} is the rate constant for diffusion. AB represents the pol β –DNA complex. The formation of the complex can be described as:

$$d[AB]/dt = (k_a[A][B] - k_d[AB]) \quad (3)$$

and the equilibrium affinity constant (K_A) for the pol β –DNA complex can be calculated based on k_a/k_d .

For the blunt-end DNA duplex, since there was no single-strand region, the interaction was presumably due to the binding of the 31 kDa domain to the dsDNA region [24,27] (Figure 2B). For the interaction between pol β and gapped DNA, the nick in the designed substrate was only 8 nt long (Figure 1C), which means that the binding of pol β to this single-stranded region alone was not feasible because a gap of 16 nt is required for the intact pol β to bind at a low protein concentration [31]. Therefore, the binding of pol β to the gapped DNA was assumed to fit the model in which the 8 kDa domain binds to the ssDNA region and the 31 kDa domain binds to the template–primer region near either the 3'-end of the upstream primer or the 5'-end of the downstream primer [7,8] (Figure 2C).

For pol β binding to DNA template–primer duplexes (dsGCPM-1–6; Figure 1D), a heterogeneous ligand 1:2 (parallel binding) model was proposed as the DNA substrates contain both a template–primer binding site and a sufficiently long single-strand region (Figure 2D). The interaction between pol β and template–primer DNA was assumed to involve (i) pol β binding to the DNA duplex region, in which the 8 kDa domain binds to the single-stranded template and the 31 kDa domain binds to the duplex in the vicinity of the 3' end of the primer, and (ii) the intact pol β binding to the DNA template overhang region, in which 16 nt are occluded by the protein (Figure 2D). The kinetic equations describing the parallel binding model can be written as:

$$d[A]/dt = k_{mt}([A]_{\text{solution}} - [A]) - (k_{a1}[A][B_1] - k_{d1}[AB_1]) - (k_{a2}[A][B_2] - k_{d2}[AB_2]) \quad (4)$$

$$d[B_1]/dt = -(k_{a1}[A][B_1] - k_{d1}[AB_1]) \quad (5)$$

$$d[B_2]/dt = -(k_{a2}[A][B_2] - k_{d2}[AB_2]) \quad (6)$$

where A represents pol β , B_1 and B_2 represent the DNA duplex binding site and the ssDNA template binding site, respectively. AB_1 is the complex with pol β bound to the dsDNA region and AB_2 is the complex with pol β bound to the ssDNA template.

The formation of these two complexes (AB_1 and AB_2) can be described by the following rate equations:

$$d[AB_1]/dt = (k_{a1}[A][B_1] - k_{d1}[AB_1]) \quad (7)$$

$$d[AB_2]/dt = (k_{a2}[A][B_2] - k_{d2}[AB_2]) \quad (8)$$

In the parallel binding model, the sensor response, R , is the total

Figure 2 Binding modes between human pol β and different DNA substrates

Simplified models showing proteolytic domains of DNA pol β interacting with different DNA substrates. (A) An ssDNA in which 16 nt are occluded by the enzyme to form a (pol β)₁₆ complex. (B) A blunt-end DNA in which only the 31 kDa domain is involved in binding of the double-stranded region. (C) A gapped DNA containing an 8 nt nick in the middle of a 50 bp duplex in which intact pol β could bind to the gapped region and only the 31 kDa domain could bind to the double-stranded region. (D) A template–primer duplex in which the 8 and 31 kDa domains are shown to bind in the vicinity of the 3' end of the primer and a single-stranded segment of the template.

Figure 3 Sensorgrams for DNA pol β binding to ssDNA and dsDNA substrates

Binding of various concentrations (20–80 nM) of DNA pol β to sensor surfaces modified with (A) ssDNA, (B) blunt-end dsDNA and (C) gapped dsDNA at 25 °C. The residual plots of fitting to the Langmuir model are also shown.

response of pol β binding to both the template–primer DNA duplex and ssDNA template. The equilibrium affinity constants for the dsDNA–pol β complex (AB_1), K_{A1} , and the ssDNA–pol β complex (AB_2), K_{A2} , can be calculated based on k_{a1}/k_{d1} and k_{a2}/k_{d2} , respectively. The contributions of AB_1 (x_1) and AB_2 (x_2) to pol β binding to the DNA template–primer duplex can be

estimated from the maximum binding capacity for these two complexes ($R_{\max1}$ and $R_{\max2}$), respectively, from the following equations:

$$x_1 = R_{\max1} / (R_{\max1} + R_{\max2}) \quad (9)$$

$$x_2 = R_{\max2} / (R_{\max1} + R_{\max2}) \quad (10)$$

Table 1 Kinetics and affinities of human pol β binding with different DNA substrates

DNA substrate	k_a ($M^{-1} \cdot s^{-1} \times 10^5$)	k_d ($s^{-1} \times 10^{-3}$)	K_A ($M^{-1} \times 10^8$)	χ^2
ssDNA	5.32 ± 0.25	4.26 ± 0.11	1.25 ± 0.07	0.617
dsDNA	7.48 ± 0.16	9.89 ± 0.18	0.76 ± 0.02	0.95
Gapped DNA	5.36 ± 0.21	6.28 ± 0.17	0.85 ± 0.01	0.989

Immobilization of DNA substrates and specific binding of pol β to ssDNA

The DNA substrates were immobilized on the sensor chip surface using biotin-streptavidin chemistry, with 5'-biotin-labelled template as the anchor. Biotinylated DNA targets bound rapidly to the immobilized streptavidin molecules on the SA5 chip and could not be washed away by HSM buffer. Control experiments were carried out using an unlabelled DNA duplex to verify the specificity of the streptavidin-biotinylated DNA interaction. No association was observed ($\Delta RU \approx 0$), indicating that non-specific adsorption of DNA on to the SA5 surface was negligible. The amount of DNA immobilized could be controlled by adjusting the concentration and injection time. Different amounts of immobilized DNA were tested for pol β binding, and optimized surface density was determined for high protein-binding capacity and low steric hindrance. For all experiments, similar amounts of DNA substrates (about 2500 RU) were immobilized on to the sensor surfaces for polymerase-binding measurements.

Different concentrations of pol β were injected on to the DNA-modified sensor surface, and the binding of these various concentrations of pol β to ssDNA was monitored by the biosensor. The net responses are shown in Figure 3(A). For example, the injection of 80 nM pol β on to the ssDNA-modified surface caused an increase of about 200 RU in 3 min, resembling a typical reversible binding reaction. A similar response in the opposite direction was observed when the pol β was washed away with the TSB buffer. Na^+ ions (100 mM) were present in the TSB buffer to facilitate pol β binding [44]. It has been shown that pol β -DNA interactions are due to the binding of two helix-hairpin-helix motifs of pol β , one in the 31 kDa domain and the other in the 8 kDa domain, in the presence of metal ions such as Na^+ [44]. No dNTP or Mg^{2+} ions were present in the solution, in order to prevent the elongation of the terminal bases of the primer by the polymerase in the cases of template-primer and gapped DNA duplexes. It can be seen that the overall sensor responses increased as the injection time and the concentration of pol β increased, indicating that an increasing amount of pol β was bound to the immobilized DNA.

CD and fluorescence studies of human pol β indicated that the 8 kDa domain has high affinity ($10^6 M^{-1}$) for ssDNA [26–28,31]. It has been shown that pol β formed a complex with ssDNA in which 16 nt were occluded by the enzyme at low protein concentration, called the $(pol \beta)_{16}$ complex, and in which the 31 kDa catalytic domain was also involved [31]. Because the concentration range of pol β used in this study was low (20–100 nM), a 1:1 DNA-pol β complex would form upon binding of pol β to the immobilized ssDNA. The binding data were fitted to the Langmuir binding model (eqns 1–3) and the residual plots for all concentrations showed that the fitting was adequate, based on the lower χ^2 (0.617) and the randomness of the residual plots. The k_a and k_d values derived from the fitting procedure and the K_A of human pol β binding to ssDNA are listed in Table 1.

Figure 4 Binding of DNA pol β to DNA template-primer duplexes containing various numbers of mismatch(es)

Different concentrations (20–100 nM) of DNA pol β were injected over the sensor surface modified with (A) dsGCPM-1, (B) dsGCPM-2, (C) dsGCPM-3, (D) dsGCPM-4, (E) dsGCPM-5 and (F) dsGCPM-6 DNA duplex at 25 °C.

From the SPR measurements, pol β binds to ssDNA with a dissociation equilibrium constant of ≈ 8 nM, which is significantly lower than the value of $1 \mu M$ obtained from the steady-state fluorescence titration study, with the same NaCl concentration (100 mM), carried out by Rajendran et al. [31]. The difference may be due to the fact that the two techniques were used under completely different conditions: solution versus solid/liquid interface, and low temperature (10 °C) [31] versus room temperature (25 °C in the present study). Therefore, in subsequent the discussion, the biosensor data are only used to compare among a set of experiments carried out under identical conditions and are not intended as absolute measurements of interactions under physiological conditions.

Pol β binding to blunt-end and gapped DNA

Similar to the ssDNA experiment, various concentrations of pol β (20–100 nM) in TSB buffer were injected over the sensor surfaces modified with dsDNA (Figure 3B). In the case of the blunt-end DNA duplex, it was assumed that only the 31 kDa fragment of pol β was involved in the binding of the double-stranded region of the DNA substrate. The biosensor results confirmed that pol β could bind with dsDNA [24,27]. The Langmuir binding model (eqns 1–3) was used for data fitting. The residual plots of the fitting and the average reduced χ^2 value (0.95) confirmed that the model was adequate in describing the binding between pol β and the immobilized dsDNA. The k_a and k_d values derived from the fitting procedure and the K_A of human pol β binding to the dsDNA are listed in Table 1.

The above results show that both ssDNA and dsDNA bound to pol β , and that pol β had a higher affinity for ssDNA than for

Figure 5 Comparison of model fitting to DNA pol β and singly mismatched DNA interactions locally and simultaneously

The residual plots for the fitting of the interaction between DNA pol β and singly mismatched DNA to the Langmuir binding and parallel models are shown. (A) dsGCPM-2; (B) dsGCPM-3. The parallel model was concluded to be a good model for curve fitting, based on the low χ^2 values (2.35 for dsGCPM-2 and 0.408 for dsGCPM-3) and the random distributions of residual plots.

dsDNA. The K_A for the ssDNA–pol β interaction ($1.25 \times 10^8 \text{ M}^{-1}$) is about 2-fold higher than that for the blunt-end dsDNA–pol β interaction ($7.56 \times 10^7 \text{ M}^{-1}$). This is consistent with the results obtained from the previous fluorescence titration study [27] showing that the affinity of intact pol β for dsDNA is 2-fold lower than that for ssDNA. This difference in binding affinity was attributed to the difference in the binding modes of pol β towards various DNA substrates. The binding of pol β with ssDNA involves both the N-terminal 8 kDa domain and the C-terminal 31 kDa domain at low protein concentration [31], whereas only the larger C-terminal fragment is involved in the binding to dsDNA [27,28]. However, because the C-terminal 31 kDa domain contributes most of the binding energy, the difference between

the equilibrium binding constants for ssDNA and dsDNA is relatively small.

An intermediate conformation between ssDNA and blunt-end dsDNA is nicked and gapped DNA. We prepared gapped DNA containing an 8 nt single-stranded region in the middle of a 50 bp duplex (Figure 1C). Pol β could bind with the gapped DNA in two binding modes: at the gapped region and at the double-stranded region (Figure 2C). Figure 3(C) shows an overlaid plot of pol β binding to gapped DNA with increasing protein concentration, indicating that pol β has affinity for duplex DNA with gaps bearing 3' and 5' termini. However, it is unclear whether pol β binds at the gapped site or the dsDNA region or both. The binding sensorgrams of pol β with the gapped DNA

Table 2 Binding kinetics and affinities of human pol β binding with different DNA template–primer duplexes (dsGCPM-1–6)

Simultaneously the data were fitted locally with parallel reaction (2:1) models with mass-transfer effect.

DNA duplex	k_{a1} ($M^{-1} \cdot s^{-1} \times 10^5$)	k_{d1} ($s^{-1} \times 10^{-2}$)	K_{A1} ($M^{-1} \times 10^7$)	x_1 (%)	k_{a2} ($M^{-1} \cdot s^{-1} \times 10^5$)	k_{d2} ($s^{-1} \times 10^{-3}$)	K_{A2} ($M^{-1} \times 10^8$)	x_2 (%)	χ^2
dsGCPM-1	9.47 ± 0.06	0.43 ± 0.01	26.2 ± 0.63	83	4.41 ± 0.02	2.56 ± 0.01	1.7 ± 0.01	17	0.886
dsGCPM-2	6.52 ± 0.04	1.88 ± 0.01	3.46 ± 0.25	68	6.6 ± 0.02	2.95 ± 0.01	2.24 ± 0.01	32	2.35
dsGCPM-3	1.84 ± 0.03	2.26 ± 0.02	0.81 ± 0.02	62	6.37 ± 0.12	1.2 ± 0.01	5.31 ± 0.11	38	0.408
dsGCPM-4	1.65 ± 0.07	3.62 ± 0.19	0.46 ± 0.03	62	6.75 ± 0.19	1.53 ± 0.05	4.41 ± 0.18	38	1.54
dsGCPM-5	1.61 ± 0.05	3.02 ± 0.1	0.53 ± 0.02	54	6.63 ± 0.17	1.7 ± 0.04	3.89 ± 0.14	46	2.86
dsGCPM-6	1.16 ± 0.03	2.56 ± 0.08	0.45 ± 0.02	44	3.96 ± 0.13	2.7 ± 0.61	2.7 ± 0.61	56	1.39

could be fitted adequately to the Langmuir binding model. The use of the parallel 1:2 binding model (eqns 4–8) did not improve the fit. Values of k_a , k_d and K_A for human pol β binding to the gapped DNA derived from the Langmuir model are listed in Table 1.

The kinetic rate constants and the affinity constants for pol β binding to dsDNA and gapped DNA were very similar. There are two possible explanations for this: (i) pol β binds to both the gapped site and the dsDNA with similar affinity and kinetics, or (ii) pol β binds to the dsDNA region only because the ssDNA region in the gapped DNA substrate is too small for pol β binding.

Pol β binding to DNA template–primer duplexes containing mismatches

We prepared various DNA template–primer duplexes containing a 50-mer template hybridized with a 15-mer primer. Mismatch(es) were introduced at or near the 3'-end of the primer, creating different conformations of DNA in that region. Because the preferred binding site of pol β is a DNA template–primer duplex, the changes in the binding-site conformation induced by the mismatches were expected to result in changes in the binding interaction of pol β with the DNA substrates. Previous studies [17,28] of pol β have postulated its mechanism of binding to DNA template–primer duplexes. The model involves the 31 and 8 kDa domains binding to the single-stranded segment of the template and in the vicinity of the 3' end of the primer (Figure 2D). Since a template overhang (35-mer) was present in the DNA substrates, this overhang region can provide sufficient space for pol β to bind. Various concentrations of pol β (20–100 nM) in TSB buffer were injected sequentially over the sensor surfaces modified with various template–primer DNA duplexes containing various numbers of mismatches (dsGCPM-1–6). The overlaid plots for the pol β binding to all DNA duplexes are summarized in Figure 4. The results indicate that pol β bound to the immobilized DNA duplexes regardless of whether mismatch(es) were present near the 3' terminus of the primer. However, the overall association and dissociation profiles for the binding of pol β to DNA duplexes containing fully matched (dsGCPM-1) or partially mismatched (dsGCPM-2–6) primers are clearly different (Figure 4).

The sensorgrams were fitted with both the Langmuir model and the parallel binding model. Figure 5 shows the residual plots of fitting of the interaction between a series of pol β solutions and the immobilized duplex DNA (dsGCPM-2 and dsGCPM-3) using both models. For the parallel model, the fitting was carried out using numerical integration analysis with multiple sets of initial values. As shown in Figure 5, the parallel model offered much better fits to the sensorgrams at all pol β concentrations than the Langmuir model. The reduced χ^2 values obtained from

the parallel model were 2.35 for dsGCPM-2 and 0.408 for dsGCPM-3, while those obtained from the Langmuir model were 10.5 for dsGCPM-2 and 1.41 for dsGCPM-3. The fitting of the sensorgrams for all DNA template–primer duplexes showed that the parallel binding model was the most adequate, based on the residual plots and χ^2 values (Table 2).

As shown in Table 2, two sets of kinetic parameters were resolved from the 2:1 binding model in the case of pol β binding to fully matched DNA (dsGCPM-1). The affinity constants of the two binding modes had the same order of magnitude. However, the fractions (x) of these two binding modes, x_1 and x_2 , were significantly different (83 and 17 %, respectively; Table 2). As pol β binds to both the template–primer region and the template-overhang region, the two different binding modes can be assigned to the interactions between pol β and the ssDNA template and those between pol β and the 3'-end of the template–primer. We assigned k_{a1} , k_{d1} and K_{A1} values for the pol β –template–primer interaction and k_{a2} , k_{d2} and K_{A2} values for the pol β –ssDNA interaction. In other words, pol β binds preferentially to the template–primer region of the fully matched DNA duplex. The rationale is that the larger fraction (x_1) related to the contribution of the major binding mode of the DNA substrate, and that k_{a2} , k_{d2} and K_{A2} values were closer to those determined for the pol β –ssDNA interaction. Subsequent experiments confirmed the assignment.

A mass-transfer step was included in all models to account for the diffusion process. The k_{mt} values for different DNA substrates were all in the order of 10^7 – 10^8 s $^{-1}$, about two orders of magnitude higher than the association rate constants (k_{a1} and k_{a2}), indicating that the systems were not limited by mass transfer.

The binding of pol β to DNA containing one, two or three mismatches provides the basic kinetic information that is the key to understanding how pol β discriminates between matched and mismatched DNA. The results showed that the introduction of a single mismatch at the first position (dsGCPM-2), second position (dsGCPM-3) and third position (dsGCPM-4) of the 3'-end of the primer decreased k_{a1} by 2-, 5- and 6-fold and increased k_{d1} by 4-, 5- and 8-fold, respectively, resulting in a significant decrease in the binding affinity of pol β to the DNA template–primer region, K_{A1} , by 8-, 32- and 56-fold, respectively. When two (dsGCPM-5) and three (dsGCPM-6) mismatches were introduced at the 3'-ends of the primers, k_{a1} was decreased by 6- and 8-fold, respectively, and k_{d1} was increased by about 6–7-fold, resulting in a decrease in K_{A1} of 50- and 58-fold, respectively. The weaker binding affinity of pol β towards the template–primer region with mismatched bases may be due to the absence of proofreading exonuclease activity from this simple polymerase.

In addition to its effect on the rate constants and binding affinity of pol β binding to the DNA template–primer region, a decrease in the binding fraction towards DNA template–primer

duplexes was also observed with the increasing number of mismatches. For fully matched DNA, the fraction of pol β binding to the double-stranded region was 83 %, whereas one (dsGCPM-2) and two (dsGCPM-5) mismatches at the 3'-terminus of the primer decreased the binding of DNA to the template-primer duplex by 15 and 30 %, respectively. Duplexes containing three consecutive mismatches (dsGCPM-6) showed $\approx$ 40 % binding with pol β in the template-primer region.

The presence of mismatch(es) caused a significant change in the binding affinity of pol β towards the dsDNA template-primer region, but there was no effect on its binding affinity to the single-stranded template overhang. The results showed that the k_{a2} , k_{d2} and K_{A2} values matched the values obtained from the ssDNA experiment. Furthermore, no obvious trend in k_{a2} , k_{d2} and K_{A2} of pol β was observed with respect to the introduction of mismatch(es). Although there was no significant effect on the kinetic rate constants and binding affinities, the fraction of pol β binding to this single-stranded region increased from 17 to 56 % when the number of mismatch(es) increased (Table 2).

Conclusion

In this study, we have used the SPR biosensor technique to characterize and obtain quantitative information on the kinetics of binding interactions between human DNA pol β and different DNA substrates. The biosensor technique allows the monitoring of binding interactions in real time and offers an alternative approach for the measurement of thermodynamic and kinetic profiles. SPR kinetic measurements revealed that pol β bound to ssDNA with a higher affinity than to blunt-end dsDNA, because of differences in both binding and dissociation rates. Pol β bound to template-primer duplexes at two sites, one in the template-primer region and the other in the region of the single-stranded template overhang. Although the association and dissociation rates of pol β for ssDNA and fully matched template-primer duplexes were different, the binding affinity constants were close to each other (of the order of $\times 10^8 \text{ M}^{-1}$). This can be explained by the similar binding orientations of the protein on these two DNA targets, in which both the 8 and 31 kDa domains of pol β were involved in the interaction. The polymerase bound preferentially to the template-primer region (83 %) when the primer strand was complementary to the DNA template. With the introduction of a single mismatch at one of the first three positions of the primer terminus, the binding affinities of pol β towards the double-stranded region decreased by 8–57-fold, with 62–68 % binding. However, after introducing three consecutive mismatches at the primer end, the binding affinity and binding fraction of pol β towards the template-primer duplex decreased 58-fold and to about 40 % respectively, indicating that the protein bound to the double-stranded region with a lower affinity and in larger amounts to the single-stranded template overhang. Thus we can conclude that pol β can identify mispaired DNA even though it does not contain any exonuclease proofreading activity.

This work was supported by the Research Grants Council, Hong Kong (CityU GERG Project no. 9040277).

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Received 24 July 2001/14 September 2001; accepted 1 November 2001