Kinetics of human polymerase β binding to gapped DNA substrates having single stranded (ss) DNA gaps with five or two nucleotide residues in the ssDNA gap has been examined, using the fluorescence stopped-flow technique. The mechanism of the recognition does not depend on the length of the ssDNA gap. Formation of the enzyme complex with both DNA substrates occurs by a minimum three-step reaction, with the bimolecular step followed by two isomerization steps. The results indicate that the polymerase initiates the association with gapped DNA substrates through the DNA-binding subsite located on the 8-kDa domain of the enzyme. This first association step is independent of the length of the ssDNA gap and is characterized by similar rate constants for both examined DNA substrates. The subsequent, first-order transition occurs at the rate of \(600-1200\) s\(^{-1}\). This is the major docking step accompanied by favorable free energy changes in which the 31-kDa domain engages in interactions with the DNA. The 5′-terminal PO\(_4\) group downstream from the primer is not a specific recognition element of the gap. However, the phosphate group affects the enzyme orientation in the complex with the DNA, particularly, for the substrate with a longer gap.

Polymerase β is one of a number of recognized DNA-directed polymerases of the eukaryotic nucleus (1–5). A characteristic feature of human pol β \(^2\) is a “simplified” set of activities. The enzyme lacks intrinsic accessory activities, such as 3′ or 5′ exonuclease, endonuclease, dNMP turnover, and pyrophosphorylisis (1, 5–7). This limited repertoire of activities reflects the very specialized function of the enzyme in human cell repair machinery which includes the gap filling synthesis involved in mismatch repair, the repair of monofunctional adducts, UV damaged DNA, and abasic lesions in DNA (1, 4, 5–12). Human pol β is a single polypeptide of \(39,000\) kDa. The crystallographic structures of both rat and human pol β have been determined at 3.6- and 2.3-Å resolution (13–15). The feature that distinguishes the pol β structure from other polymerases is the presence of a small 8-kDa domain connected with the tip of the fingers through a tether of 14 amino acids (13, 16). The domain possesses the enzymatic ability to catalyze the release of the 5′-terminal deoxyribose phosphate residue from the incised apurinic-apirimidinic site that is a common intermediate product in base excision repair (12). The active site of the DNA synthesis resides predominantly in the large 31-kDa domain of the enzyme (13–15).

A puzzling problem in the DNA recognition mechanism, by a DNA repair polymerase, is the fact that the enzyme must recognize the damaged DNA, containing a small ssDNA gap, in the context of the large excess of the dsDNA. Although the catalytic properties of the domains are established, the role of both domains in the recognition of the DNA substrates is just now emerging (17–20). Quantitative equilibrium studies have shown that both human and rat pol β bind the ssDNA in two binding modes (17, 20). The binding modes differ in the number of occluded nucleotide residues and have been referred to as the (pol β)\(_{16}\) and (pol β)\(_{5}\) binding modes (19–23). The existence of the two binding modes is a consequence of the presence of the two structural domains of the protein possessing the DNA-binding subsites, with different DNA binding capabilities (17, 20). Both DNA-binding subsites form the total DNA-binding site of the polymerase. In the (pol β)\(_{16}\) binding mode, both the 8- and 31-kDa domains are involved in interactions with the ssDNA, i.e. the total DNA-binding site is engaged in interactions with the nucleic acid. In the (pol β)\(_{5}\) binding mode, only the 8-kDa domain is engaged in interactions with the DNA (17, 20). The subsite located on the 8-kDa domain has similar affinity for both ss and dsDNA, while the subsite on the 31-kDa domain seems to have a preference for the dsDNA, although this has not been rigorously proven.

The mechanism of the formation of the (pol β)\(_{16}\) and (pol β)\(_{5}\) binding modes, by human pol β, is a complex, multiple-step sequential process (21). In both ssDNA-binding modes the formation of the protein-nucleic acid complex is initiated through the DNA-binding subsite located on the 8-kDa domain (21). Thus the DNA-binding subsite on the 8-kDa domain plays the role of the initiation-binding site of the enzyme to the ssDNA. Analysis of the kinetic data revealed that transitions to subsequent intermediates are also generated through interactions at the 8-kDa domain-DNA interface resulting in the engagement of the 31-kDa domain in interactions with the nucleic acid. The DNA-binding initiation role of the subsite located on the 8-kDa domain is reflected in its energetically homogeneous structure and the capability of accommodating DNA oligomers of different lengths with similar affinity (22).

In the base-excision repair processes, human pol β fills the ssDNA gaps formed in the damaged DNA (1, 5, 9). Thus, the physiologcal substrates for the enzyme are gapped DNAs that have a stretch of ssDNA embedded between the primer and the dsDNA downstream from the primer. Thermodynamic studies of human pol β binding to the gapped DNA substrates indicate that the ability of the 8-kDa domain DNA-binding site to interact with different nucleic acid conformations is crucial for anchoring the enzyme on the gap (19, 20). In these complexes, the 8-kDa domain binds to the ss and/or dsDNA part of the
DNA, downstream from the primer, depending on the length of the ssDNA gap. The 31-kDa domain of the enzyme associates with the dsDNA part of the gapped DNA substrate that contains the primer. Thus, similar to the formation of the (pol β)α binding mode, engagement of the entire total DNA-binding site of the enzyme provides a large increase of the affinity for the specific recognition of the gapped DNA structure.

Understanding the mechanistic aspects of the pol β-gapped DNA recognition process is of great importance for elucidation of the polymerase mechanism at the molecular level. Such analysis will also provide important insights into the recognition mechanisms of specific DNA substrates by other nucleic acid-dependent polymerases. The fundamental questions that arise here are the following. How does the mechanism of the gapped DNA recognition differ from the mechanisms of the enzyme binding to the ssDNA in its different binding modes? What is the formation rate of the different intermediates? What are the energetics of the conversions between the different intermediates? How does the length of the ssDNA gap and the presence of the 5’-terminal phosphate group affect the mechanism? Is there a particular step that makes a dominant contribution to the recognition process? Despite its paramount importance, the direct analysis of the dynamics of the gapped DNA substrate recognition by human pol β has not been quantitatively addressed before.

In this article, we report the stopped-flow kinetic analyses of human pol β interactions with the gapped DNA substrates that differ by the number of the nucleotide residues in the ssDNA gap. We provide direct evidence that the mechanism of the specific gap complex formation by human pol β is a three-step, sequential reaction. The bimolecular step includes a very fast association with the DNA, through the 8-kDa domain, followed by two docking steps. The dynamics of the bimolecular step is independent of the length of the ssDNA gap. The internal transition, directly following the bimolecular step, is the major docking step, and is characterized by a large, favorable free energy change for the examined gapped DNA substrates. In this step the DNA-binding subsite located on the 31-kDa domain engages in interactions with the DNA. The 5’-terminal PO₄ group downstream from the primer does not guide the polymerase to the gap, although it stabilizes the first intermediate in the case of the gapped DNA substrate with a longer gap. The data indicate that the phosphate group affects the enzyme orientation in the complex with the DNA and the structure of the ssDNA gap in the complex with the enzyme.

MATERIALS AND METHODS

Reagents and Buffers—All solutions were made with distilled and deionized >18 MΩ (Milli-Q Plus) water. All chemicals were reagent grade. Buffer C is 10 mM sodium cacodylate adjusted to pH 7.0 with HCl, 10% glycerol, 0.1 mM EDTA-Na₂. The temperature and concentrations of NaCl and MgCl₂ in the buffer are indicated in the text.

Human Polymerase β—Human pol β was purified as previously described (17–20). The concentration of the protein was determined using the extinction coefficient, ε₂₈₀ = 2.1 × 10⁴ cm⁻¹ M⁻¹, determined by the approach based on the Edelhoch method (23–27).

Nucleic Acids—The ssDNA oligomers were purchased from Midland Certified Reagents (Midland, TX). Oligomers were at least >95% as judged by autoradiography on polyacrylamide gels. The fluorescent residue is introduced through the fluorescein phosphoramidite, i.e. the label replaces one of the bases in the oligomers. The concentration of the nucleic acids was determined as previously described by us (28, 29).

Fluorescence Measurements—Steady-state fluorescence titrations were performed using the SLM-AMINCO 8100 spectrophotofluorometer (26–30). The fractional fluorescence increase of the nucleic acids is defined as ΔF = (Fᵢ - Fᵢ₀)/Fᵢᵢ₀, where Fᵢ is the value of the fluorescence at a given titration point and Fᵢ₀ is the initial fluorescence of the sample before addition of the protein (31).

Stopped-flow Kinetics—Fluorescence stopped-flow kinetic experiments were performed using the SX.MV18 stopped-flow instrument.

![Fig. 1. The two gapped DNA substrates that are used to examine the interactions of human pol β with the gapped DNA.](Image)

where F(t) is the fluorescence intensity at time t, F(∞) is the fluorescence intensity at t = ∞, Aᵢ is the amplitude corresponding to ith relaxation process, λᵢ is the time constant (reciprocal relaxation time) characterizing the ith relaxation process, and n is the number of relaxation processes. All further analyses of the data were performed using Mathematica (Wolfram, Urbana, IL) and KaleidaGraph (Synergy Software, PA) on Macintosh G3 and G4 computers.

Analysis of Kinetic Data—Analyses of the stopped-flow kinetic data have been performed using the matrix projection operator technique (21, 32–36). This approach is particularly useful in analyzing the amplitudes of the studied reactions and has been extensively described by us (21, 32–36).

RESULTS

Formation of the Gap Complex between Human Pol β and Gapped DNA—The gapped DNA substrates used to examine the mechanism of the gapped DNA recognition by human pol β are depicted in Fig. 1. The substrates contain two dsDNA parts,
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Each having 10 base pairs (bp). The primary structures of the dsDNA parts are identical in all gapped DNA substrates. The ssDNA gap separates the dsDNA parts. The gap has two, or five residues, i.e. the substrates differ by three residues in the gap. This difference corresponds to the lowest estimate of the site-size of the (pol β)n binding mode (5 ± 2) that the enzyme forms with the ssDNA (17–20). The bases of the nucleotide residues in the ssDNA gap are all adenosines with the exception of the residue at the 5′ end of the gap that is replaced by fluorescein. It provides the signal to monitor the binding and kinetics. Analogous gapped DNA substrates containing the 5′-terminal phosphate group downstream from the primer are included in Fig. 1.

Our previous studies have shown that binding of the enzyme to the etheno derivatives of the considered gapped DNAs is accompanied by a nucleic acid fluorescence increase (19–21). However, the observed signal is not adequate enough to quantitatively examine the complex kinetics of the reaction. On the other hand, we have found that binding of the enzyme to the gapped DNA, containing the fluorescein residue in place of one of the nucleotide residues in the ssDNA gap, as shown in Fig. 1, is accompanied by a large fluorescence increase of the DNA. This signal change provides the required resolution to monitor the kinetics of the enzyme-gapped DNA complex formation. In this context, fluorescein derivatives of the DNA, where the label is substituted for one of the bases, could be very useful in examining other protein-nucleic acid interactions. We have used analogous ssDNA oligomers containing the fluorescein residue in our analyses of the kinetics of the polymerase binding to the ssDNA (21).

At maximum saturation, the considered gapped DNA substrates, depicted in Fig. 1, can bind up to three molecules of human pol β (20). However, the binding process is very complex. A single enzyme molecule forms a high affinity complex that includes the ssDNA gap, while the remaining two polymerase molecules are bound with a significantly lower affinity to two dsDNA parts. We refer to the high affinity complex as the gap complex (20, 21). The high affinity of the gap complex results from the fact that only in this complex the enzyme engages both the 8- and 31-kDa domains in interactions with the nucleic acid. The 8-kDa domain interacts with the dsDNA part of the 5′ end of the template strand, downstream from the primer, while the 31-kDa domain engages in interactions with the dsDNA part at the 3′ end of the template strand which includes the primer (Fig. 1). The large difference between the affinities of the two binding processes strongly separates them with respect to the protein concentration (20). This strong separation of the two binding processes allows us to study both the thermodynamics and kinetics of the specific human pol β binding directly to the gap and the formation of the gap complex, independently from the nonspecific enzyme binding to the dsDNA parts.

Fluorescence titration of the gapped DNA substrate, containing five residues in the ssDNA gap (Fig. 1, substrate A), with human pol β in buffer C (pH 7.0, 10 °C), containing 100 mM NaCl and 1 mM MgCl2, is shown in Fig. 2a. The binding curve has been analyzed using a single-binding site isotherm,

$$\Delta F = \Delta F_{\text{max}} \left( \frac{K_{G5}[\text{human pol } \beta]}{1 + K_{G5}[\text{human pol } \beta]} \right)$$

(Eq. 2)

where $\Delta F_{\text{max}}$ is the maximum relative fluorescence increase and $K_{G5}$ is the overall equilibrium binding constant characterizing the enzyme affinity for the gapped DNA substrate, i.e. the gap complex with five residues in the ssDNA. The solid line is the computer fit of the experimental isotherm to Equation 2, with $K_{G5} = (1 \pm 0.2) \times 10^9$ M$^{-1}$ and $\Delta F_{\text{max}} = 0.63 \pm 0.05$. It is clear that the theoretical line provides an excellent description of the experimental isotherm indicating that the formation of the gap complex is exclusively observed (20). In other words, in the examined protein concentration range, binding of the additional enzyme molecules to the dsDNA parts of the DNA substrate, with the affinities characterized by binding constants in the range of $10^{-5}$–$10^{-6}$ M$^{-1}$, does not occur (21).

A 5′-terminal phosphate group, downstream from the primer in the damaged DNA is a common intermediate product in base excision repair (1, 9, 12). Fluorescence titration of the gapped DNA substrate containing five residues in the ssDNA gap and also the 5′-terminal phosphate group (Fig. 1, Substrate C), with human pol β in buffer C (pH 7.0, 10 °C), containing 100 mM NaCl and 1 mM MgCl2, is included in Fig. 2a. The binding curve has been analyzed using a single-binding site isotherm, defined by Equation 2, that provides $K_{G5} = (1 \pm 0.2) \times 10^9$ M$^{-1}$ and $\Delta F_{\text{max}} = 1.76 \pm 0.05$. Thus, the presence of the 5′-terminal PO₄
group has no effect on the overall affinity (20). However, the PO$_4^-$ group has a dramatic effect on the fluorescence change accompanying the formation of the complex with $\Delta F_{\text{max}}$ being by a factor of $\sim 3$ higher than that observed in the absence of the phosphate group (Fig. 2). Such a large difference in the spectroscopic properties of the polymerase-DNA complex, in the absence and presence of the PO$_4^-$ group, indicates that the conformations of the nucleic acid, particularly the structures of the ssDNA gap in both complexes, are significantly different. This is despite the fact that the affinities are virtually identical.

Because the fluorescein residue may affect the affinities of human pol $\beta$ for the examined gapped DNA substrates, we performed fluorescent titrations in the presence of the competing non-fluorescent gapped DNA substrate with five residues in the ssDNA gap (Fig. 1), but containing adenosine in place of the fluorescein residue. The corresponding competition titration curves, for the gapped DNA without and with 5'-terminal phosphate group, are included in Fig. 2a. The solid lines are computer fits of the experimental isotherms, which provide binding constant, $K_{\text{binding}} = (1 \pm 0.2 \times 10^{9} \text{M}^{-1})$, for both unmodified DNAs (30, 31). Thus, the presence of the fluorescein residue does not affect, to any detectable extent, the affinity of the protein for the gapped DNA substrate independently of the presence of the phosphate group (see below).

Fluorescence titration of the gapped DNA substrate, containing two residues in the ssDNA gap (Fig. 1, Substrate B), with human pol $\beta$ in buffer C (pH 7.0, 10 °C), containing 100 mM NaCl and 1 mM MgCl$_2$, is shown in Fig. 2b. The solid line is the computer fit of the experimental isotherm using the equation analogous to Equation 2, with $K_{\text{binding}} = (3 \pm 0.3 \times 10^{9} \text{M}^{-1}$ and $\Delta F_{\text{max}} = 0.73 \pm 0.05$. Thus, the overall binding constant is by a factor of $\sim 3$ higher for the gapped DNA substrate with a smaller gap (21). Also, the value of the maximum relative fluorescence increase, $\Delta F_{\text{max}} = 0.73 \pm 0.05$, is higher than the value of 0.63 $\pm$ 0.05 obtained for the DNA substrate with five residues in the gap, indicating differences in the structure of the ssDNA gap between two DNA substrates in the complex with the polymerase (Fig. 2a).

Fluorescence titration of the gapped DNA substrate, containing two residues in the ssDNA gap and the 5'-terminal phosphate group, downstream from the primer (Fig. 1, Substrate D), with human pol $\beta$ in buffer C (pH 7.0, 10 °C), containing 100 mM NaCl and 1 mM MgCl$_2$, is included in Fig. 2b. The solid line is the computer fit of the experimental isotherm (Equation 2), with $K_{\text{binding}} = (3 \pm 0.3 \times 10^{9} \text{M}^{-1}$ and $\Delta F_{\text{max}} = 1.5 \pm 0.07$. Similar to the DNA substrate with five residues in the gap the presence of the phosphate group does not, to a detectable extent, affect the overall affinity of the enzyme for the substrate with the smaller gap. However, the maximum relative fluorescence, $\Delta F_{\text{max}}$, increase is by a factor of $\sim 3$ higher than the corresponding parameter obtained in the absence of the phosphate group. Thus, similar to the DNA substrate with a long ssDNA gap, the presence of the 5'-terminal PO$_4^-$ group strongly affects the conformation of the protein-nucleic acid complex, although to a smaller extent. Also, competition titrations of the unmodified DNA substrates, containing two adenosines in the ssDNA gap, show that the fluorescein residue in the gap does not affect, to any extent, the affinity of the enzyme for the DNA (data not shown).

**Kinetics of Human Pol $\beta$ Binding to the Gapped DNA Substrate with Five Residues in the ssDNA Gap**—The fluorescence stopped-flow kinetic trace of the gapped DNA substrate, having the ssDNA gap with five residues, after mixing $1.5 \times 10^{-8}$ m nucleic acid with $1.6 \times 10^{-7}$ m human pol $\beta$ (final concentrations) in buffer C (pH 7.0, 10 °C), containing 100 mM NaCl and 1 mM MgCl$_2$, is shown in Fig. 3a. The plot is shown in logarithmic scale with respect to time. The initial horizontal part of the trace corresponds to the steady-state fluorescence intensity, recorded for $\sim 2$ ms before the flow stopped, b, the same fluorescence stopped-flow trace as in a, together with the zero line trace (lower trace), which is obtained after mixing the nucleic acid, at the same concentration as used with the protein, but only with the buffer. The solid line is the same three-exponential nonlinear, least-square fit of the experimental curve as shown in a.

The stopped-flow kinetic curve, together with the trace corresponding to the DNA substrate alone, at the same concentration of the nucleic acid as used with the protein, but only mixed with the buffer, is shown in Fig. 3b. Recall, the total amplitude of the reaction, $A_{\text{Total}}$, is the difference between the fluorescence intensity recorded at the end point of the kinetic trace and the zero line recorded for the nucleic acid alone (32–36). Therefore, although the two-exponential fit provides an excellent description of the observed kinetic process, it yields the sum of the amplitudes that is larger than the observed total amplitude of the overall, relaxation process. This behavior is observed at all studied enzyme concentrations (data not shown). In other words, these data indicate that the observed reaction contains at least one additional step, charac-
Scheme I, with the relative fluorescence intensities $F$ clearly indicates that this relaxation time characterizes an observed dependence of the relaxation times upon the pol concentration. Therefore, the simplest mechanism which can describe the formation of the gap complex between human pol and dsDNA part of the gapped DNA substrate would begin to interfere with the observed kinetics (20). However, as we discussed above, the relaxation process characterized by the relaxation time, $\tau_1$, is preceded by a faster process, characterized by $\tau_2$. Therefore, the presence of the initial, very fast process indicates that $\tau_2$ characterizes an intramolecular transition. The seemingly linear dependence of $\tau_1$ upon the enzyme concentration examined only the initial part of the functional dependence of $1/\tau_2$ upon [enzyme] is recorded (21, 32–35). On the other hand, the independence of $1/\tau_3$ of the polymerase concentration clearly indicates that this relaxation time characterizes another intramolecular transition of the complex (21, 32–35). Therefore, the simplest mechanism which can describe the observed dependence of the relaxation times upon the pol concentration and the presence of the third fast step is a three-step, sequential reaction in which bimolecular binding is followed by two first-order transitions described by the Scheme I.

$$
\begin{align*}
\text{Human pol } \beta + \text{gapped DNA} &\rightleftharpoons (G) \rightleftharpoons (G) \rightleftharpoons (G) \\
&\text{at } k_1, k_2, k_3 \\
&\text{at } k_{-1}, k_{-2}, k_{-3}
\end{align*}
$$

**SCHEME I**

Although the relaxation time for the first, fast normal mode cannot be extracted from the data, the amplitude of the first mode, $A_1$, can be obtained as a difference between the total amplitude of the reaction, $A_{Tot}$, and the known amplitudes of the second and third normal modes, $A_2$ and $A_3$ (32–36) as the following equation.

$$
A_1 = A_{Tot} - A_2 - A_3 
$$

(Eq. 3)

Fig. 4c shows the dependence of the normalized, individual amplitudes, $A_1$, $A_2$, and $A_3$, of the three relaxation steps upon the human pol $\beta$ concentration. The amplitude of the first relaxation step, $A_1$, is negative. Also, its absolute values slightly increase with increasing concentrations of human pol $\beta$. The positive amplitude, $A_2$, dominates the relaxation process over the entire examined range of [human pol $\beta$]. The values of $A_2$ slightly increase with the increasing of the [enzyme] and at high [human pol $\beta$] are larger than 1, i.e., the amplitude is larger than the total amplitude $A_{Tot}$. This results from the fact that the values of $A_2$ compensate for the negative values of $A_1$ (21). The values of $A_3$ are also positive and have little dependence on the enzyme concentration. The observed behavior of the individual amplitudes as functions of the human pol $\beta$ concentration is in agreement with the proposed mechanism defined by Scheme I (21).

To extract all rate and spectroscopic parameters characterizing the partial steps and the intermediates of the kinetic system, we applied the following strategy utilizing the fact that both the amplitudes and the relaxation times contain information about the examined system (32–35). The analysis is initiated by numerical nonlinear, least-square fitting of the individual relaxation times. Because the first step is very fast, the fits were performed with the starting value of the rate constant, $k_1$, near the diffusion-controlled limit, e.g., $1 \times 10^{10}$ M$^{-1}$ s$^{-1}$. We also know the value of the macroscopic binding constant, $K_{GR} = (1 \pm 0.2) \times 10^9$ M$^{-1}$, for the enzyme binding to the
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The obtained rate constants for each step provide partial equilibrium steps by,

$$K_{eq} = K_1 + K_2 + K_3$$

(Eq. 4)

where $K_1 = k_1/k_{-1}$, $K_2 = k_2/k_{-2}$, and $K_3 = k_3/k_{-3}$. The above relationship reduces to five the number of independent parameters in fitting the relaxation times. Subsequently, the obtained rate constants were used as starting values in the fitting of the three individual amplitudes and extract relative molar fluorescence parameters, i.e., to assess the conformational state of the protein-nucleic acid complex in each intermediate. This was accomplished using the matrix projection operator technique (32–36). This part of the analysis uses the value of the maximum fractional increase of the nucleic acid fluorescence accompanying the complex formation, $\Delta F_{max} = 0.6 \pm 0.03$, that is known from independent equilibrium titrations (Fig. 2a).

The $\Delta F_{max}$ parameter can be analytically expressed (32–35) as,

$$\Delta F_{max} = \frac{\Delta F_2}{1 + K_2 + K_3} + \frac{K_2 \Delta F_3}{1 + K_2 + K_3} + \frac{K_3 \Delta F_4}{1 + K_2 + K_3}$$

(Eq. 5)

where $\Delta F_2 = (F_2 - F_1)/F_1$, $\Delta F_3 = (F_3 - F_1)/F_1$, and $\Delta F_4 = (F_4 - F_1)/F_1$ are fractional fluorescence intensities of each intermediate in the formation of the gap complex, relative to the molar fluorescence intensity of the free DNA substrate, $F_1$. It should be pointed out that, contrary to the $\Delta F$ values, the fluorescence parameters, $F_2$, $F_3$, and $F_4$, are relative molar fluorescence intensities, but not fractional intensities, with respect to the free nucleic acid fluorescence. Equation 5 provides an additional relationship among the fluorescence parameters, thus, decreasing the number of independent variables. The refinement of the values of rate constants and molar fluorescence parameters was accomplished by global fitting that simultaneously includes all relaxation times and amplitudes. The solid lines in Fig. 4, a–c, are computer fits of the relaxation times and amplitudes, according to the above mechanism, using a single set of rate and spectroscopic parameters.

The obtained rate constants and relative molar fluorescence intensities for the mechanism defined by Scheme I are included in Table 1.

The forward rate constant, $k_1 = 6 \times 10^8$ m$^{-1}$ s$^{-1}$, characterizing the bimolecular binding step is very high, close to the value predicted for the diffusion-controlled reaction (37, 38) (see "Discussion"). The value of the rate constant, $k_{-1} = 1000 \pm 100$ s$^{-1}$, indicates that the enzyme can easily be released back to the solvent from the first intermediate (G1). The transition to the second intermediate, (G2b), is also a fast process with the forward rate constant, $k_2 = 1150 \pm 120$ s$^{-1}$ (Table I). However, the transition to (G3) is dramatically slower with the forward rate constant, $k_{-3}$, being $\sim 4$ orders of magnitude lower than $k_2$. The obtained rate constants for each step provide partial equilibrium constants $K_1 = (6 \pm 1.4) \times 10^6$ m$^{-1}$, $K_2 = 77 \pm 26$, and $K_3 = 1.4 \pm 0.6$ (Table I). Thus, the first step has a predominant contribution to the free energy of the enzyme binding to the examined gapped DNA substrate, $\Delta G^*$ (Fig. 1, Substrate A). Notice that the second step also provides a significant contribution to the $\Delta G^*$. The (G3) to (G4) transition is much less energetically favorable (see "Discussion").

Amplitude analysis indicates that the formation of the first intermediate (G1) is not accompanied by any molar fluorescence change of the DNA substrate ($F_2 = 1 \pm 0.03$), as compared with the free nucleic acid (Table I). However, the formation of the subsequent (G2b) intermediate is accompanied by a molar fluorescence increase ($F_3 = 1.55 \pm 0.05$), indicating different structures of the protein-nucleic acid complex in both

**Table I**

| Gap Structure | $K_1$ | $K_2$ | $K_3$ | $K_{eq}$ |
|---------------|-------|-------|-------|---------|
| **NPO**      | 5     | 5     | 50    | 5       |
| **PO**       | 5     | 5     | 50    | 5       |

*Values relative to the fluorescence, $F_1$, of the free gapped DNA substrates (details in text).
intermediates (see “Discussion”). Conformational transition to 
(G)3 induces a very low, additional molar fluorescence increase 
of the nucleic acid over $F_3$ ($F_4 = 1.64 \pm 0.05$), indicating that 
the structure of the examined protein-gapped DNA complex is 
similar in both (G)2 and (G)3 intermediates.

Kinetics of Human pol β Binding to the Gapped DNA Sub- 
strate with Two Residues in the ssDNA Gap—Analogous 
stopped-flow kinetic studies have been performed with gapped 
DNA substrate with two residues in the ssDNA gap (Fig. 1, 
Substrate B). The experiments have been performed in the 
same solution conditions, i.e., buffer C (pH 7.0, 10 °C) containing 
100 mM NaCl and 1 mM MgCl₂. The experimental kinetic traces required a two-exponential fit (data not shown). However, 
the two-exponential fit yields the sum of amplitudes signif-
icantly larger than the total amplitude resulting from the com-
plex formation at all examined enzyme concentrations. Thus, as 
we discussed above, the data indicate that there is at least one 
additional very fast step preceding the observed trace, charac-
terized by the relaxation time, $\tau_1$, and negative amplitude.

The reciprocal relaxation times, $1/\tau_2$ and $1/\tau_3$, for the associ-
ation of human pol β with the gapped DNA substrate, with two 
residues in the ssDNA gap (Fig. 1, Substrate B), as functions of 
the total human pol β concentration, are shown in Fig. 5, a and b. The dependence of the amplitudes upon [human pol β] is 
shown in Fig. 5c. The values of $1/\tau_2$ show a more pronounced 
hyperbolic dependence upon [human pol β] than observed in 
the case of the DNA gapped with a longer gap (Fig. 4a) which results from the higher affinity of the enzyme for the 
DNA with a shorter gap. This behavior and the existence of the 
preceding fast process indicate that the relaxation time, $\tau_2$, 
describes an intramolecular isomerization. The values of $1/\tau_3$ 
are independent of the [enzyme], an indication that this relaxation 
time characterizes another intramolecular transition 
(32–35, 38). Therefore, association of human pol β with the 
gapped DNA having two residues in the gap, is described by the 
same mechanism as depicted by Scheme I.

The analysis of the experimental stopped-flow data has been 
performed as described above. The obtained kinetic and 
spectroscopic parameters, obtained from the examination of the 
data shown in Fig. 5, are included in Table I. The rate constant, 
$k_2$ and $k_{-1}$, of the bimolecular step are very similar to the rate 
constants determined for the association of the enzyme with 
the gapped DNA substrate with five residues in the gap (Table I). 
Thus, the dynamics of the initial binding step are not af-
fected by the large difference in the size of the ssDNA gap, 
although the structure of the (G)1 intermediate is different 
from the analogous intermediate in the enzyme association 
with the DNA substrate with five residues in the ssDNA gap, 
as indicated by the significantly lower value of $F_3$. On the other 
hand, the value of the forward rate constant, $k_2 = 600 \text{ s}^{-1}$, is 
lower as compared with the value of $k_2 = 1150 \text{ s}^{-1}$ observed for 
the DNA substrate with five residues in the ssDNA gap. Thus, 
the lower size of the gap hinders the transition to the (G)2 
intermediate in the human pol β-gapped DNA complex. How-
ever, the value of $k_{-2}$ is also lower by a factor of $3$, as 
compared with the DNA substrate with the longer gap. As a 
result, the partial equilibrium constant, $K_{\beta}$, assumes a higher 
value, indicating that the (G)2 intermediate is energetically 
more favorable for the DNA substrate with only two residues in 
the gap. Nevertheless, the very similar values of the relative 
molar fluorescence intensities of the (G)2 intermediate for both 
gapped DNA substrates indicate that the structure of this 
intermediate is similar, i.e. independent of the length of the 
ssDNA gap (Table I). A pronounced effect of the size of the 
ssDNA gap is observed on the transition to the third interme-
deicate that the structure of this intermediate is similar, i.e. independent of the length of the 
ssDNA gap (Table I). A pronounced effect of the size of the 
ssDNA gap is observed on the transition to the third interme-
diate, (G)3. Both $k_4$ and $k_{-3}$ are larger by a factor of $20$, i.e. 
the process becomes much faster in both directions with a larger 
increase toward the formation of (G)3, resulting in a larger
value of $K_3$ for the gapped DNA substrate with a shorter gap. The (G)$_3$ intermediate for this DNA substrate becomes a significant part of the population of the enzyme–DNA complex at equilibrium.

Effect of the 5'-Terminal Phosphate Group Downstream from the Primer on the Kinetics of Human pol β Binding to Gapped DNA Substrates—The presence of the 5'-terminal phosphate group, downstream from the primer, plays an important role in DNA substrate recognition and catalysis (11, 14). However, direct thermodynamic analysis of the effect of the 5'-terminal phosphate on the binding of human pol β to DNA substrates shows only a moderate effect on the overall binding constant of the enzyme for the gap (Fig. 2b) (20). The role of the 5'-terminal phosphate group in the dynamics of the gapped DNA recognition process by human pol β has been examined using the gapped DNA substrates shown in Fig. 1, but with the 5'-terminal phosphate group on the oligonucleotide downstream from the primer (Fig. 1, Substrates C and D).

The experimental stopped-flow kinetic traces require a two-exponential fit for both DNA substrates, with five and two residues in the ssDNA gap (data not shown). Moreover, the sum of the observed amplitudes is significantly larger than the total amplitudes resulting from the complex formation for all examined enzyme concentrations. Thus, as we discussed above, the data indicate that there is at least one additional very fast step preceding the observed trace, characterized by the relaxation time, $\tau_1$, that must be characterized by negative amplitude. The reciprocal relaxation times, $1/\tau_2$ and $1/\tau_3$, for the association of human pol β with the gapped DNA substrate with five residues in the ssDNA gap and the 5'-terminal phosphate group downstream from the primer (Fig. 1, Substrate C) as functions of the total human pol β concentration, are shown in Fig. 6, a and b. The dependence of the observed amplitudes upon the [human pol β] is shown in Fig. 6c. In the examined [human pol β] range the values of $1/\tau_2$ show typical, hyperbolic dependence upon [human pol β] and clearly indicate that the relaxation time, $\tau_2$, describes an intramolecular isomerization. Similar to the gapped DNAs discussed above, independence of $1/\tau_2$ upon the enzyme concentration clearly indicates that this relaxation time characterizes another intramolecular transition (21). Therefore, association of human pol β with the considered gapped DNA, having the 5'-terminal phosphate downstream from the primer, is described by the mechanism defined by Scheme I. Analogous behavior of the relaxation times and amplitude has been observed for the gapped DNA substrate with only two residues in the ssDNA gap (data not shown). The obtained kinetic and spectroscopic parameters for both DNA substrates are included in Table I.

The obtained data indicate that the forward rate constant, $k_1$, of the bimolecular step is not affected by the presence of the phosphate group, independently of the size of the ssDNA gap. However, the value of $k_{-1}$ is lower by a factor of $\sim 3$ for the DNA substrate with five residues in the ssDNA gap, i.e. the presence of the phosphate group increases the stability of the first intermediate, (G)$_1$, as compared with the corresponding DNA substrate without the 5'-terminal phosphate group (Table I). The presence of the phosphate group has a profoundly different effect on the formation of the second intermediate, (G)$_2$, for both gapped DNAs. In the case of the DNA molecule with five residues in the ssDNA gap, the forward rate constant, $k_3$, is lower by a factor of $\sim 4$. Thus, the presence of the phosphate group hinders the transition to the (G)$_2$ intermediate for a longer gap. The phosphate group also decreases the values of $k_{-2}$ as a result, the value of the partial equilibrium constant, $K_2$, is decreased by a factor of $\sim 2$. Thus, the dynamics of the (G)$_1 \rightarrow$ (G)$_2$ transition and the free energy change accompanying the formation of the (G)$_2$ intermediate are affected by the PO$_4$ group. In the case of the gapped DNA substrate with
two residues in the ssDNA gap, both rate constants, \( k_a \) and \( k_{-a} \), are not changed by the presence of the phosphate group (Table I). Within experimental accuracy, the value of the partial equilibrium constant, \( K_{eq} \), is the same as observed in the absence and presence of the PO_4 group. Similarly, little effect of the phosphate group is observed for the (G)_2 \( \leftrightarrow \) (G)_3 transition. Thus, the presence of the PO_4 group has no effect on either the dynamics or the free energy changes accompanying the formation of the (G)_2 and (G)_3 intermediates when the gap is only two residues long.

**DISCUSSION**

The Mechanism of the Gap Complex Formation by Human pol \( \beta \) Is a Multiple Step Kinetic Process—Elucidation of the dynamics of human pol \( \beta \) interactions with gapped DNA substrates is of paramount importance for understanding the gapped DNA recognition process by the enzyme at the molecular level. The kinetic data obtained in this work indicate that the mechanism of the gap complex formation with the DNA substrates with two and five residues in the ssDNA gap is a minimum three-step, sequential process in examined solution conditions described by Scheme I. Thus, the kinetic mechanism of the gap complex formation is not affected by the large difference between the sizes of the ssDNA gap. The independence of the mechanism of the gap recognition of the size of the gap provides the first indication that the size of the ssDNA gap does not constitute any specific recognition element for the enzyme. However, the bimolecular step is followed by at least two conformational transitions of the enzyme-ssDNA complex that differ dramatically in the values of the rate constants and accompanying free energy changes. Analysis of the entire mechanism provides the clue for understanding the specific formation of the gap complex.

The bimolecular step of the gap complex formation by human pol \( \beta \) is very fast and close to the diffusion-controlled reaction (32–41). The determined forward rate constant, \( k_1 \approx 6 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \), is high and independent of the length of the ssDNA gap. The independence of \( k_1 \) upon the length of the gap is fully understandable in the context of our recent studies of the kinetics of human pol \( \beta \) binding to the ssDNA in its (pol \( \beta \))_16 and (pol \( \beta \))_5 binding modes (21). These data indicate that, in the bimolecular step, the enzyme exclusively associates with the ssDNA, using its 8-kDa domain. Thus, the first initial contact between the enzyme and the nucleic acid occurs through the DNA-binding subsite of the 8-kDa domain. Moreover, thermodynamic studies of the 8-kDa domain interactions with the ssDNA for the analogous rat pol \( \beta \) showed that the domain has very similar affinities for both ss- and dsDNA (22). The DNA-binding subsite of the domain can accept ss and ds oligomers of a different length with similar affinity. Notice, the site size of the 8-kDa domain with the ssDNA is \( \sim 10 \) nucleotide residues and is \( \sim 10 \) bp with the dsDNA (22). Therefore, in any complex with the examined gapped DNA substrates (Fig. 1), the domain predominantly engages a similar nucleic acid structure, i.e. a mixture of ss and dsDNA conformations. Thus, the initial contact with any gapped DNA substrate occurs through the same DNA-binding subsite of the enzyme that does not differentiate between different DNA conformations resulting in very similar values of \( k_1 \), as observed in the case of the ssDNA (21). The similarity of the (G)_1 intermediates is also indicated by the similar values of the dissociation rate constant, \( k_{-1} \), which indicates the same lifetime of the (G)_1 intermediate for both gapped DNA substrates.

The similarity between the first step in the formation of the (pol \( \beta \))_3 and (pol \( \beta \))_2 binding modes, where the 8-kDa domain of the polymerase makes the first contact with the nucleic acid, and the first step in the binding of the enzyme to the gapped DNA substrate is also evident in the kinetics of the isolated 8- and 31-kDa domains binding to the DNA.\(^2\) Analogous kinetic studies indicate that the first step in the binding of the isolated 8-kDa domain to the ss and dsDNA is characterized by rate constants, \( k_1 \approx 5 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \) and \( k_{-1} \approx 1000 \text{ s}^{-1} \), which are very close to the same parameters observed for the intact enzyme binding to the gapped DNA (Table I). The affinity of the 31-kDa domain for the DNA is much lower than that of the 8-kDa domain. The shortest relaxation time appears only at the protein concentration range that is more than an order of magnitude higher than that observed for the 8-kDa domain. This behavior strongly suggests that the first step in the 31-kDa domain is much slower than observed for the 8-kDa domain, and the intact enzyme. Thus, these data additionally reinforce the conclusion that the enzyme makes the first contact with the DNA using its 8-kDa domain.

Despite the similar dynamics and energetics of the first binding step for both gapped DNAs, the amplitude analysis shows a large difference in the nucleic acid fluorescence changes accompanying the formation of the (G)_1 intermediates between the two nucleic acids (Tables I). In the case of the gapped DNA substrate with five residues in the gap, the molar fluorescence intensity is, within experimental accuracy, the same as the molar fluorescence of the free nucleic acid. On the other hand, formation of the (G)_1 intermediate, in the case of the gapped DNA with only two residues in the ssDNA gap, is accompanied by a large fluorescence decrease. These data strongly suggest that the very fast conformation transition of the enzyme–DNA complex, in the formation of the intermediate, depends on the length of the ssDNA gap. Conformational transitions of ss nucleic acid oligomers, particularly base stacking, are very fast processes which occur in the range of microseconds (39–42). Thus, most probably, the observed difference in the molar fluorescence intensities results from the differences in the induced changes in stacking interactions between the bases in the ssDNA gaps of different sizes (see below).

**Anchoring of Human pol \( \beta \) on the Gapped DNA Occurs in the Second Kinetic Step**—The first step in the mechanism of human pol \( \beta \) binding to the gapped DNA provides the dominant contribution to the free energy of binding, independently of the length of the ssDNA gap (Table I). However, the value of the obtained partial equilibrium constant, \( K_1 \approx 6 \times 10^8 \text{ M}^{-1} \), is in the same range as the binding constant for the nonspecific binding of the enzyme to the dsDNA previously determined by us (20). Therefore, the strong specific recognition of the gapped structure of the nucleic acid over the dsDNA does not occur in the first binding step. However, once the enzyme associates with the gapped DNA, the first intermediate, (G)_1, undergoes a transition to a (G)_2 intermediate. In the absence of a 5’ terminal phosphate group, the forward rate constant, \( k_2 \), is in the range of \( \sim 630–1150 \text{ s}^{-1} \), for both gapped DNA substrates. Thus, the transition is fast, particularly, for the longer gap. The results previously obtained with the ssDNA indicate that the analogous transition in the formation of the (pol \( \beta \))_16 binding mode by human pol \( \beta \) is generated through interactions with the 8-kDa domain with the nucleic acid (21). Notice that the value of \( k_2 \) obtained for the gapped DNAs is similar to \( k_2 \approx 570 \text{ s}^{-1} \) which was obtained for the corresponding transition in the formation of the (pol \( \beta \))_16 binding mode (21). In the context of these data and the discussion above, the similar values of \( k_2 \) for very different DNA substrates are not surprising. They indicate that in the first intermediate, (G)_1, the enzyme is bound to the nucleic acid through its 8-kDa domain and the transition to

\(^2\) M. J. Jezewska, R. Galletto, and W. Bujalowski, unpublished data.
the (G)_2 intermediate is generated at the same interface of the protein–DNA complex, i.e., the interface between the 8-kDa domain and the nucleic acid.

The most paramount feature of the transition, (G)_1 ↔ (G)_2, is that the formation of the (G)_2 intermediate is accompanied by a large favorable free energy change, \( \Delta G^\circ_{2} \), that strongly contributes to the overall free energy of binding, \( \Delta G^\circ \), for both gapped DNA substrates. This contribution is larger for the gapped DNA with only two residues in the ssDNA gap as reflected by the larger value of the partial equilibrium constant, \( K_{2} \) (Table I). The subsequent (G)_2 ↔ (G)_3 transition contributes much less to the overall \( \Delta G^\circ \). Previous thermodynamic studies indicated that, in the gap complex, human pol β engages both 8- and 31-kDa domains in the interactions with the gapped DNA (20). Moreover, the engagement of the 31-kDa domain DNA-binding subsite in interactions with the ssDNA is observed in the analogous, second step in the formation of the (pol β)_{16} binding mode (21). Therefore, the large favorable free energy change accompanying the (G)_1 ↔ (G)_2 step indicates that in this step the DNA-binding subsite located on the 31-kDa domain of the enzyme engages in interactions with the gapped DNA and provides for strong stabilization of the (G)_2 intermediate. In other words, in this intermediate, the entire total DNA-binding site of human pol β becomes involved in the complex with the nucleic acid and the enzyme anchors on the gap (20, 21).

In the absence of the 5'-terminal phosphate group, the larger value of the equilibrium constant, \( K_{2} \), for the gapped DNA substrate with only two residues in the ssDNA gap, results, predominantly, from a significantly lower value of the backward rate constant \( k_{-2} \) (Table I). Such an increased stability of the (G)_2 intermediate indicates a more favorable orientation of the polymerase in the complex with the DNA with a shorter gap, where the dsDNA containing the primer is in close proximity to the 31-kDa domain. The effect of the length of the gap is particularly visible in the dynamics and energetics of the third step that, in the absence of the 5'-terminal phosphate group, is an order of magnitude faster and energetically more favorable for the shorter gap. The data indicate that the human pol β-gapped DNA complex, already more stable in the case of the shorter gap, is additionally stabilized with the shorter ssDNA gap.

Although the dynamics and energetics of the (G)_2 ↔ (G)_3 transition depends strongly on the length of the ssDNA gap, conformations of the ssDNA gap in the (G)_2 and (G)_3 intermediates are very similar for both DNA substrates, i.e., independent of the length of the ssDNA gap, as indicated by very similar and large molar fluorescence intensities (Table I). Our previous studies using etheno derivatives of the gapped DNA substrates indicated that formation of the gap complex with human pol β is accompanied by a significant increase of the nucleic acid fluorescence (17, 20, 21). Because the fluorescence increase of the etheno derivatives of the nucleic acid results predominantly from the separation and immobilization of the bases, such a fluorescence change indicates that binding of the enzyme induces an immobilization and separation of bases in the ssDNA gap (17, 20, 21). Thus, the large increase of the relative molar fluorescence of the (G)_2 and (G)_3 intermediates suggests that the fluorescence increase of the fluorescein residue results from the immobilization and separation of the bases in the ssDNA gap in the complex with the enzyme. Very similar values of the relative molar fluorescence of the (G)_2 and (G)_3 intermediates strongly suggest that the ssDNA gap has similar conformations in both intermediates for both gapped DNA substrates in the complex with the enzyme. In other words, the conformational adjustment of the DNA and the protein in the (G)_2 ↔ (G)_3 transition does not affect the structure of the ssDNA of the gap. In this context, the significant difference in dynamics and energetics, particularly for the (G)_2 ↔ (G)_3 transition, between the gapped DNA substrates, with different lengths of the ssDNA gaps, strongly suggests that the (G)_2 ↔ (G)_3 step includes changes in protein interactions at the interfaces of the 8- and 31-kDa domains of the enzyme rather than changes in the structure of the gap.

It is interesting that, in the absence of the 5'-terminal phosphate group, the values of the rate constants, \( k_{+2} \) and \( k_{-3} \), for the DNA substrate with five residues in the gap, are significantly lower than the proposed values of the rate of nucleotide incorporation by pol β (6 s^{-1}) (43). These results strongly suggest that the enzyme is already properly oriented in the (G)_2 intermediate in the complex with this gapped DNA to initiate the catalysis. A fast and energetically favorable transition to (G)_3, in the case of the DNA molecule with only two residues in the gap, indicates that both (G)_2 and (G)_3 should be catalytically competent with this gapped DNA substrate. A similar structure of both (G)_2 and (G)_3 intermediates also supports this conclusion. In other words, human pol β may be able to initiate DNA synthesis from different intermediates depending on the length of the ssDNA gap. We are currently examining these processes.

The 5'-Terminal Phosphate Group Does Not Guide the Polymerase to the Gap, but the PO_4 Group Affects the Orientation of the Enzyme in the Gap Complex—The 5'-terminal phosphate group, downstream from the primer in the damaged DNA, is a common intermediate product in base excision repair (1, 9, 12). Thus, the phosphate group could provide a specific structural element of the gapped DNA substrate that distinguishes it from the surrounding dsDNA and could be specifically recognized by the polymerase. Intuitively, such a specific recognition should affect the dynamics of the enzyme association, particularly in the first binding step. The kinetic data described in this work indicate that the forward rate constant, \( k_{1} \), is not affected by the 5'-terminal phosphate group for both gapped DNAs. On the other hand, in the case of the DNA substrate with five residues in the gap, the value of \( k_{-1} \) is decreased, resulting in a higher partial equilibrium binding constant, \( K_{1} \), characterizing the bimolecular binding step (Table I). The presence of the 5'-terminal phosphate group only moderately stabilizes the (G)_1 intermediate for the longer gap. The phosphate group has no effect on the dynamics and energetics of the first binding step in the enzyme association with the DNA substrate with only two residues in the gap. These data clearly indicate that the 5'-terminal PO_4 group is not a strong recognition element of the gapped DNA in the initial binding step. In other words, the 5'-terminal phosphate does not guide human pol β to the gap, independently of the size of the gap. This result may be surprising, because the binding site for the 5'-terminal phosphate is located on the 8-kDa domain of the polymerase and the domain makes the first contact with the nucleic acid (see above) (12, 21).

Recall that the 5'-terminal PO_4 group does affect the activities of pol β on gapped DNAs by changing the distributive character of the DNA synthesis, in the absence of the PO_4 group, to the processive synthesis in the presence of the PO_4 group (9). Direct thermodynamic studies clearly show that the overall affinity is only moderately, if at all, affected by the presence of the 5'-terminal phosphate group (20). Therefore the effect of the phosphate group cannot be explained by an increase of the overall ground-state affinity of the enzyme. Moreover, the kinetic data discussed above indicate that the phosphate group has little effect on the first binding step and only moderately stabilizes the (G)_1 intermediate in the enzyme complex with...
gapped DNA with a longer gap. However, the presence of the 5′-terminal PO4 group has a significant effect on the dynamics and energetics of the second and third binding steps, particularly, for the gapped DNA substrate with five residues in the ssDNA gap. In other words, the presence of the 5′-terminal phosphate group affects the internal conversions between the intermediates following the binding step. Such an effect indicates that the 5′-terminal phosphate group plays a role in inducing specific orientations of the already bound enzyme.

Amplitude analysis indicates that molar fluorescence intensities of the (G)1 and (G)2 intermediates are significantly higher than the analogous parameters obtained in the absence of the phosphate group (Table I). As we discussed above, the increase of the nucleic acid fluorescence indicates that the bases in the ssDNA gap are additionally separated and immobilized in the presence of the 5′-terminal phosphate group. These data indicate that the presence of the 5′-terminal phosphate induces strong structural changes of the ssDNA gap, particularly, in the case of the DNA substrate with five residues in the gap. The presence of the phosphate group makes the dynamic and structural parameters, characterizing the polymerase binding to the gapped DNA substrate with five residues in the gap, more similar to the same parameters observed for the gapped DNA with a shorter gap (Table I).

The kinetic and structural data discussed in this work provide insight as to how the gap recognition process may induce change from the distributive character of the DNA synthesis on the DNA substrates with longer gaps, to the processive synthesis in the presence of the 5′-terminal phosphate group (9). First, the backward rate constants, k_–1 and k_–2, are decreased in the presence of the phosphate group, i.e. the lifetime of the (G)_1 and (G)_2 intermediate is increased. Second, a large increase in the rigidity of the template strand in the ssDNA gap, as well as the larger separation of the bases, can facilitate the dNTP binding and recognition. In turn, this will lead to the increased processivity of the enzyme action on the longer ssDNA gaps.

*Model of the Gapped DNA Recognition by Human pol β*—As we mentioned above, the most puzzling problem in the gapped DNA recognition mechanism by a DNA repair polymerase is the fact that the enzyme must recognize the DNA fragment, containing a small ssDNA gap, in the context of the large excess of the dsDNA. Equilibrium studies provided the first direct evidence that, with the ssDNA longer than ~16 nucleotide residues, human pol β forms the (pol β)_1-binding mode where both DNA-binding subsites located on the small 8-kDa domain and on the large catalytic 31-kDa domain, respectively, are engaged in interactions with the nucleic acid (17, 20). Analogously, in the formation of the human pol β complex with the gapped DNAs, the 8-kDa domain associates with the dsDNA downstream from the primer, while the 31-kDa domain engages the dsDNA part that contains the primer (17, 20).

The mechanism of the gapped DNA recognition by a DNA repair polymerase must be sequence independent, but it must be specific for the structure of the DNA substrate. Kinetic analyses of human pol β binding to gapped DNA substrates indicate a plausible model for the gapped DNA recognition by human pol β in the context of the surrounding dsDNA and the involvement of both DNA-binding subsites in particular steps of the recognition process. The model is schematically depicted in Fig. 7. In the initial binding step, the enzyme associates the nucleic acid using its DNA-binding subsite located on the 8-kDa domain. This is a nonspecific step characterized by the rate constants, k_s and k_–s. The step is very fast, electrostatically driven, and includes a conformational rearrangement of the nucleic acid (base stacking) and/or protein leading to the formation of the (G)_1 intermediate. Subsequently, the enzyme probes the conformation of the bound DNA by engaging the DNA-binding subsite located on the 31-kDa domain in interactions with the nucleic acid. Thus, probing of the proper orientation includes interactions of both DNA-binding subsites of the enzyme with the DNA. The transition is also very fast, allowing the polymerase to test several orientations with respect to the ssDNA gap. If the polymerase is in an improper orientation, with respect to the gap, or is bound to a non-gapped DNA, the formed complex dissociates back to the (G)_1 intermediate where it exchanges rapidly with the free enzyme in solution. The kinetic results obtained with the ssDNA indicate that the dissociation rate constant, k_–2, for a nonspecific DNA substrate, is in the range of ~600 s⁻¹, resulting in a low partial equilibrium constant, K_g, is of the order of 100. The conformational change of the DNA substrate in this step includes separation and immobilization of the nucleic acid bases in the ssDNA gap. However, the crystal structures of the enzyme-DNA co-complex indicate that structural changes include the DNA bending in the total DNA-binding site of the enzyme (13–15). The transition to the (G)_2 intermediate (k_1 and k_–1) is a structural change of the enzyme-ssDNA complex facilitated by the lower length of the ssDNA gap. The transition to the (G)_2 intermediate is the final docking step of the gapped DNA in the total binding site on the polymerase. It is very probable that other polymerases, particularly DNA repair polymerases, use a similar multiple-step docking mechanism in their interactions with DNA substrates. Thus, the analysis of the mechanism of the promoter DNA recognition by the T7 RNA polymerase indicated the presence of three binding steps with different contributions to the total free energy of binding (44). It should be noted that the DNA-binding site of most polymerases is structurally and functionally heterogeneous, analogously to the structural and functional heterogeneity of the total DNA-binding site of human pol β (16).
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