Energetics and Specificity of Rat DNA Polymerase β Interactions with Template-primer and Gapped DNA Substrates*

Received for publication, November 17, 2000, and in revised form, January 24, 2001
Published, JBC Papers in Press, January 25, 2001, DOI 10.1074/jbc.M010434200

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Interactions between rat polymerase β (pol β) and the template-primer, as well as gapped DNAs, were studied using the quantitative fluorescence titration technique. Stoichiometries of rat pol β complexes with DNA substrates are much higher than stoichiometries predicted by the structures of co-crystals. The data can be understood in the context of the two single-stranded (ss)DNA-binding modes of the enzyme, the (pol β)16 and (pol β)5 binding modes, which differ by the number of nucleotides occluded by the protein. The 8-kDa domain of the enzyme engages the double-stranded (ds)DNA downstream from the primer, while the 31-kDa domain has similar affinity for the ss-ds DNA junction and the dsDNA. The affinity of rat pol β for the gapped DNA is not affected by the size of the gap. The results indicate a plausible model for recognition of the gapped DNA by rat pol β. The enzyme binds the ss-ds DNA junction of the gap using the 31-kDa domain. This binding induces an allosteric transition, resulting in the association of the 8-kDa domain with the dsDNA, leading to an amplification of the affinity for the gap. The 5' terminal phosphate, downstream from the primer, has little effect on the affinity, but affects the ssDNA conformation of the gap.

Polymerease β is one of a number of recognized DNA-directed polymerases of the eukaryotic nucleus (1–5). The enzyme has a very specialized function in mammalian cell repair machinery. The activities of pol β have been proposed to include the gap filling synthesis involved in mismatch repair (4, 6–8), in the repair of monofunctional adducts, UV damaged DNA, and abasic lesions in DNA (9–14). Thus, a processive “gap fillings” synthesis, observed in vitro, by rat pol β on gapped DNAs, is consistent with the proposed in vivo activities (2, 6–8). A characteristic feature of rat pol β is a “simplified” repertoire of activities. The enzyme lacks intrinsic accessory activities, such as 3' - or 5'-exonuclease, endonuclease, dNMP turnover, and pyrophosphorylation (1–4, 6, 7). Such limited activities reflect the very specialized function of the polymerase in the DNA metabolism.

The crystal structure of the rat pol β enzyme revealed a typical polymerase fold, a thumb, palm, and fingers, due to its resemblance to the human hand (15–17). However, what distinguishes the pol β structure from other polymerases is the presence of a small 8-kDa domain that is connected with the tip of the fingers through a tether of 14 amino acids (15–17). Solution studies showed that the 8-kDa domain has significant affinity for the single-stranded (ss)DNA, indicating that the domain plays a key role in recognition of this nucleic acid conformation, i.e. it is the template-binding domain (15, 18, 19, 21, 22). The active site of the DNA synthesis and the dsDNA affinity were proposed to reside predominantly in the large 31-kDa catalytic domain (15, 18, 19, 23).

Recently, we examined interactions of rat and human polymerase β with the ssDNA (20, 24). Our data showed that rat pol β binds the ssDNA in two binding modes which differ in the number of occluded nucleotide residues, the (pol β)16 and (pol β)5 binding mode. The binding modes differ in affinities and abilities to induce conformational changes in the ssDNA (20). Thus, the intrinsic affinity of the enzyme in the (pol β)16 binding mode is approximately an order of magnitude higher than the affinity in the (pol β)5 binding mode. On the other hand, when bound in the (pol β)5 binding mode, the enzyme induces much more profound structural changes in the ssDNA, suggesting strong base-base separation and DNA immobilization in the complex (20). The obtained results also indicate that in the (pol β)16 binding mode, both the 8- and 31-kDa domain of the enzyme, are involved in interactions with the ssDNA. In the (pol β)5 binding mode, the 8-kDa domain predominately is engaged in interactions with the nucleic acid (20).

Elucidation of the pol β-ssDNA recognition processes constitutes a first step toward understanding the molecular mechanism of the polymerase. However, in vivo, the enzyme also recognizes the more complex DNA substrates, gapped DNAs with different ssDNA gaps, and template-primer substrates resulting from the DNA damage (4, 21–23). Such interactions are certainly more complex and involve the simultaneous interactions of the polymerase with both the ss- and dsDNAs (4, 21, 22). Moreover, steady-state kinetic analysis suggests that the presence of the 5'-terminal phosphate group, downstream from the primer, amplified the enzyme affinity for the gapped DNA (22, 23). Despite the paramount importance for understanding the polymerase mechanism, the direct analysis of the energetics of rat pol β interactions with the template-primer and the gapped DNA substrates has not been quantitatively addressed before.

In this article, we report the analysis of rat pol β interactions with different DNA substrates, including the primer-template, with different ssDNA extensions and gapped DNA substrates, with different gap sizes, using the quantitative fluorescence titration technique (20). We provide direct evidence that rat pol β binds the template-primer DNA and the gapped DNA substrates with stoichiometries much higher than predicted by the crystallographic structure of the co-complexes...
with the DNA. These high stoichiometries can be accounted for by the fact that, in solution, the enzyme uses two binding modes in its interactions with the ssDNA (20). The data indicate that the 8-kDa domain of the enzyme is engaged in interactions with the dsDNA, downstream from the primer. The 5’-terminal phosphate, downstream from the primer, has little effect on the enzyme affinity although it affects the structure of the ssDNA in the complex. The affinity and stoichiometry of rat pol β complexes with gapped DNAs are not affected by the decreasing size of the ssDNA gap. A plausible model of the ssDNA gap recognition by rat pol β is proposed.

**EXPERIMENTAL PROCEDURES**

**Reagents and Buffers**—All chemicals were reagent grade. All solutions were made with distilled and deionized >18MΩ (Milli-Q Plus) water. Buffer C is 10 mM sodium cacodylate adjusted to pH 7.0 with HCl, 1 mM MgCl₂, 1 mM dithiothreitol, and 10% glycerol. The temperatures and concentrations of NaCl in the buffer are indicated throughout the text.

**Rat Polymerase**—Rat pol β was purified using a previously published procedure (14, 17, 18, 20). The concentration of the protein was determined using the extinction coefficient εₑᵥₑₑ = 2.1 × 10⁴ M⁻¹ cm⁻¹, determined by the approach based on the Edelhoch method (20, 24–27).

**Nucleic Acids**—All nucleic acids were purchased from Midland Certified Reagents (Midland, TX). The etheno-derivatives of the nucleic acids were obtained by direct synthesis using eA phosphoramidite (28, 29). Concentrations of all ssDNA oligomers have been spectrophotometrically determined, using the nearest neighbor analysis (30–32).

**Fluorescence Measurements**—All steady-state fluorescence titrations were performed as previously described by us (20, 24, 32–35). The binding was followed by monitoring the fluorescence of the ethenoadenosine residues placed in the ssDNA extensions of the template-primer or gapped DNA substrates. Computer analyses of the binding isotherms were performed using Mathematica (Wolfram, IL) or Kaleidograph (Synergy Software, Reading, PA). The relative fluorescence increase of the nucleic acid, ΔF, upon binding rat pol β is defined as 

$$
\Delta F = \frac{F - F_i}{F_i}
$$

where 

$$
F = \frac{N_{pol β}}{N_{pol β} + N_{DNA}}
$$

is the initial value of the fluorescence of the sample, and $F_i$ is the fluorescence of the sample at a given titration point. The data indicate that the binding of rat pol β to the various DNA substrates by monitoring the fluorescence increase, $ΔF$, of their etheno-derivatives upon the complex formation. The method to obtain quantitative estimates of the average degree of binding, $S_i$, (number of protein molecules bound per DNA substrate) and the free protein concentration, $P_{free}$, has been previously described in detail by us (20, 24, 28, 29, 32–35). Briefly, the experimentally observed $F$ has a contribution from each of the different possible $i$ complexes of rat pol β with a ssDNA. Thus, the observed fluorescence increase is functionally related to $S_i$ by (32–35)

$$
\Delta F = \sum_i \Delta F_{max}
$$

where $\Delta F_{max}$ is the molecular parameter characterizing the maximum fluorescence increase of the nucleic acid with rat pol β bound in complex $i$. The same value of $\Delta F$ obtained at two different total nucleic acid concentrations, $N_{DNA}$ and $N_{DNA}′$, indicates the same physical state of the nucleic acid, i.e. the degree of binding, $S_i$, and the free pol β concentration, $P_{free}$, must be the same. The values of $S_i$ and $P_{free}$ are then related to the total protein concentrations, $P_{pol β}$ and $P_{pol β}′$, and the total nucleic acid concentrations, $N_{DNA}$ and $N_{DNA}′$, at the same value of $ΔF$, by

$$
P_{pol β} = P_{pol β}′ - \frac{\sum_i N_{pol β} - N_{pol β}′}{S_i - S_i′}
$$

where $x = 1$ or $2$ (32–35).

**RESULTS**

**Binding of Rat pol β to Template-Primer DNA Substrates with 5-Nucleotide Long ssDNA Extension**—The two binding modes, (pol $β_{16}$) and (pol $β_{5}$), which rat pol β forms with the ssDNA, differ by the number of the occluded nucleotide residues in the protein-nucleic acid complex (20). To determine the effect of the structure of a DNA substrate on the energetics of the enzyme binding in different binding modes, interactions of the enzyme with the template-primer DNA have been examined using DNA substrates, in which the ssDNA extension can accommodate the enzyme in the (pol $β_{16}$) and/or the (pol $β_{5}$) binding mode. Template-primer DNA substrates, used in these studies, are depicted in Fig. 1. The duplex part of each substrate is 10 bp long and is located at the 5’ or 3’ end of the template strand. The ssDNA extensions have 5 (substrates A and B) or 15 (substrates C, D, and E) nucleotide residues which correspond to the site-size of 5 ± 2 and 15 ± 2 nucleotide residues, occluded by the enzyme in the complex with the ssDNA complex in the (pol $β_{16}$) or (pol $β_{5}$) binding mode (20). The ssDNA extensions of the template strand have a stretch of five fluorescent ethenoadenosine residues (eA) adjacent directly to the dsDNA. Substrate B has the 3’ ssDNA extension with four eAs. The presence of eAs provides the fluorescence signal to monitor the complex formation with the polymerase (30–35). Substrate E is the same as D, but contains a 5’ terminal phosphate group on the oligonucleotide in the dsDNA part at the 5’ end of the template strand.

![Fig. 1. Template-primer DNA substrates used to examine the energetics of rat pol β binding to template-primer DNAs.](http://www.jbc.org/)

The duplex part of each substrate is 10 bp long and is located at the 5’ or 3’ end of the template strand. The ssDNA extensions have 5 (substrates A and B) or 15 (substrates C, D, and E) nucleotide residues which correspond to the site-size of 5 ± 2 and 15 ± 2 nucleotide residues, occluded by the enzyme in the complex with the ssDNA complex in the (pol $β_{16}$) or (pol $β_{5}$) binding mode (20). The ssDNA extensions of the template strand have a stretch of five fluorescent ethenoadenosine residues (eA) adjacent directly to the dsDNA. Substrate B has the 3’ ssDNA extension with four eAs. The presence of eAs provides the fluorescence signal to monitor the complex formation with the polymerase (30–35).
Analogous fluorescence titrations have been performed with the template-primer substrate, containing the ssDNA extension at the 3’ end of the template (Fig. 1; substrate B) (data not shown). Similar to substrate A, the maximum relative increase of the nucleic acid fluorescence reaches the value of $1.2 \pm 0.1$ (Table I). In the first binding phase, characterized by a relative fluorescence increase of $0.6 \pm 0.1$, the degree of binding reaches the value of $1 \pm 0.2$, thus, showing that in the first step a single molecule of the polymerase associates with this template-primer substrate. In the low-affinity phase, extrapolation of $\Delta F$ to the maximum fluorescence increase, $\Delta F_{\text{max}} = 1.2 \pm 0.1$, provides a value of $\Sigma v_i = 2.3 \pm 0.2$.

The obtained stoichiometries are surprisingly high. Recall, with a template-primer DNA similar to substrate A (Fig. 1), only one rat pol β molecule has been found bound to the DNA in the crystal (16). However, the stoichiometries obtained for both substrates can be understood if one takes into account that the ssDNA extensions are long enough to accommodate only one polymerase molecule in the (pol) β$_b$ binding mode, while another enzyme molecule can associate with the dsDNA part of the DNA (16, 20). In other words, the considered DNA substrates have two different binding sites. Because all DNA substrates examined in this work (Figs. 1 and 5) have the same dsDNA part and different ssDNA extensions, the binding to the dsDNA parts should be characterized by the same affinity for all studied substrates (see below).

The two binding sites on the considered DNA substrates differ structurally. Therefore, the association of rat pol β with these sites can be analyzed as binding to two discrete binding sites (36). The partition function, $Z$, and the average degree of binding, $\Sigma v_i$, are then described as,

$$Z = 1 + (K_{\text{DS}} + K_b)P_F + K_{\text{DS}}K_bP_F^2$$

(Eq. 4)

and

$$\Sigma v_i = \frac{(K_{\text{DS}} + K_b)P_F + 2K_{\text{DS}}K_bP_F^2}{Z}$$

(Eq. 5)

where $P_F$ is the free rat pol β concentration, $K_{\text{DS}}$ and $K_b$ are the binding constants for the dsDNA part and to the ssDNA extension in the (pol) β$_b$ binding mode, respectively. The observed relative fluorescence change, $\Delta F$, of the nucleic acid fluorescence is then defined as,

$$\Delta F = \frac{(\Delta F_{\text{DS}}K_{\text{DS}} + \Delta F_bK_bP_F + (\Delta F_{\text{DS}} + \Delta F_b)K_{\text{DS}}K_bP_F^2)}{Z}$$

(Eq. 6)

where $\Delta F_{\text{DS}}$ and $\Delta F_b$ are fluorescence increases accompanying the binding of rat pol β to the dsDNA part and the ssDNA extension, respectively. The values of $\Delta F_{\text{DS}}$ and $\Delta F_b$ can be determined from the plots of the observed fluorescence change as a function of the average degree of binding, $\Sigma v_i$, and the known maximum observed fluorescence change $\Delta F_{\text{max}} = \Delta F_{\text{DS}} + \Delta F_b$ (Fig. 2; Table I).

The solid lines in Figs. 2a are computer fits using Equations 4–6 which provide $K_{\text{DS}} = (1.8 \pm 0.5) \times 10^6$ M$^{-1}$ and $K_b = (4.6 \pm 0.6) \times 10^6$ M$^{-1}$, for the DNA substrate with the 5’ ssDNA extension, and $K_{\text{DS}} = (2 \pm 0.5) \times 10^6$ M$^{-1}$ and $K_b = (3 \pm 0.6) \times 10^5$ M$^{-1}$, for the nucleic acid with the 3’ ssDNA extension, respectively (Table I). Notice, because both DNA substrates have the same dsDNA part, the values of $K_{\text{DS}}$ are virtually the same, reflecting the presence of the same binding site on both nucleic acids. The very similar values of $\Delta F_{\text{DS}}$ indicate that the presence of the protein bound to the dsDNA affects the ssDNA conformation to a similar extent in both substrates.

However, the determined values of $K_b$ differ significantly between the two nucleic acids (Table I). Previous studies indicated that rat pol β binds template-primer and gapped DNA

Fig. 2. a, fluorescence titrations ($\lambda_{\text{em}} = 325$ nm, $\lambda_{\text{exc}} = 410$ nm) of the template-primer DNA substrate (Fig. 1; substrate λ), containing the ssDNA extension with five nucleotide residues at the 5’ end of the template, with rat pol β, in buffer C (pH 7.0, 10 °C), containing 100 mM NaCl, at two different concentrations of the nucleic acid: $\bullet 2.22 \times 10^{-6}$ M; □ $4.44 \times 10^{-6}$ M. The solid lines are nonlinear least squares fits of the fluorescence titration curves, according to the model of the two binding sites, defined by Equation 6. The lines are plotted using a single set of parameters: $K_{\text{DS}} = 1.8 \times 10^6$ M$^{-1}$, $K_b = 2.6 \times 10^6$ M$^{-1}$, $\Delta F_{\text{DS}} = 0.7$, and $\Delta F_b = 0.4$ (details in text). Notice that for this substrate, $K_b$, the dependence of the relative fluorescence increase, $\Delta F$, upon the degree of binding, $\Sigma v_i$, of the rat pol β-DNA substrate complex. The values of $\Sigma v_i$, have been determined using the quantitative method described under “Experimental Procedures.” The solid lines are the limiting slopes of the two binding phases. The dashed line is an extrapolation of the degree of binding to the maximum value of the observed fluorescence increase $\Delta F_{\text{max}} = 1.1 \pm 0.1$ which provides the maximum stoichiometry of 2 ± 0.3 of the rat pol β-DNA substrate complex.
substrates in a defined orientation, with the small 8-kDa domain facing the 5′ end of the template strand and the large 31-kDa domain facing the 3′ end of the template strand (4, 11). Because both substrates differ only in the location of the dsDNA, with respect to the ssDNA extension, such a large difference between the values of $K_D$ indicates that the enzyme binds differently to the 5′ than to the 3′ extension.

In the case of the DNA substrates with the 3′ extension, rat pol β can only form the (pol β)$_b$ binding mode (20). However, $K_D$ is by factor of ~15 higher than the same parameter, $K_D = (2.1 \pm 0.7) \times 10^6$ M$^{-1}$ characterizing the affinity of the enzyme for the ssDNA oligomer, d[-pA]$_{15}$, indicating that the 8-kDa domain of the enzyme is engaging in interactions with the dsDNA part of the substrate (see below) (20). In the case of the DNA substrate with the 5′ extension, the value of $K_D$ is ~2 orders of magnitude higher than the in trinuc binding constant for the (pol β)$_b$ binding mode determined for the ssDNA oligomer (20).

The data indicate that the high affinity step must predominantly correspond to the formation of the (pol β)$_b$ binding mode or to the simultaneous binding of the pol β molecule to the ssDNA of the template and the ss-ds DNA junction of the template-primer substrate. However, the affinity of the (pol β)$_b$ binding mode formed exclusively with the ssDNA is ~2 orders of magnitude lower than the affinity of the observed first binding phase in Fig. 3b (20) (see below). Such a large difference in affinities excludes the formation of only the (pol β)$_b$ binding mode with the ssDNA extension (20). Thus, the data indicate that the high affinity step must predominantly correspond to the formation of the template-primer complex. Because the length of the ssDNA is 15 nucleotide residues, the 8-kDa domain can interact with the ssDNA of the template, while the large 31-kDa domain is engaged in interactions with the ss-ds DNA junction of the substrate (16).

The results show that, at saturation, four rat pol β molecules bind to the considered DNA substrate (Fig. 3b; Table II). Once again, such high stoichiometry is fully understandable in the context of different ssDNA enzyme binding modes and the structure of the DNA substrate (20). Notice, the ssDNA with a length of 15 nucleotide residues can accommodate, at saturation, three pol β molecules in the (pol β)$_b$ binding mode. As the enzyme concentration increases, the negative lattice entropy factor becomes very large (20, 24, 37, 38). At high pol β concentrations, the template-primer complex is replaced by the (pol β)$_b$ binding mode with three enzyme molecules associated with the ssDNA extension. The existence of mixed complexes is not possible because the formation of the template-primer complex and the binding in the (pol β)$_b$ binding mode on a stretch of ssDNA with 15 nucleotide residues is mutually exclusive (20). The ssDNA extension of the substrate cannot accommodate a rat pol β molecule bound in the template-primer complex and another one in the (pol β)$_b$ binding mode. Association of the fourth rat pol β molecule corresponds to the binding of one enzyme molecule to the dsDNA part of the template-primer substrate, as determined for the DNA substrates with the ssDNA of only 5 nucleotide residues in length (Fig. 2; Table I).

The considered interacting system is very complex. In the simplest approach, the formation of the template-primer complex and

### Table I

| Substrate | Maximum stoichiometry | $K_D$ (m$^{-1}$) | $K_S$ (m$^{-1}$) | $\Delta F_{DSS}$ | $\Delta F_{S}$ | $\Delta F_{max}$ |
|-----------|-----------------------|----------------|----------------|--------------------|----------------|------------------|
| A         | 2 ± 0.2               | (1.8 ± 0.5) $\times 10^6$ | (4.6 ± 0.6) $\times 10^6$ | 0.7 ± 0.1 | 0.35 ± 0.1 | 1.1 ± 0.1 |
| B         | 2.3 ± 0.2             | (2.0 ± 0.5) $\times 10^6$ | (3.0 ± 0.6) $\times 10^6$ | 0.6 ± 0.05 | 0.65 ± 0.1 | 1.2 ± 0.1 |

*Although included in the table as $K_D$ and $\Delta F_{max}$, these parameters characterize association of the enzyme with the ss-ds DNA junction of the substrate A and is referred in the text as $K_D$ and $\Delta F_{DSS}$, respectively (see text for details).*

The errors are standard deviations determined using three to four independent titration experiments.

#### Thermodynamic and spectroscopic parameters for rat pol β binding to the template-primer DNA substrates, which have ssDNA extensions with 5 nucleotide residues (Fig. 1; substrates A and B) in buffer C (pH 7.0, 10 °C) containing 100 mM NaCl
binding to the dsDNA part of the substrate are independent, and can be described by a partial partition function, $Z_1$, as,

$$Z_1 = 1 + (K_{TP} + K_{DS})P_F + K_{TP}K_{DS}P_F^2$$  \hspace{1cm} (Eq. 7)

where $K_{TP}$ and $K_{DS}$ are binding constants characterizing the formation of the template-primer complex and binding to the dsDNA part, respectively. As the protein concentration increases, the template-primer complex is replaced by the (pol $\beta_5$) binding mode while binding to the dsDNA remains unaffected. The part of the total partition function, which describes the binding of rat pol $\beta$ to the (pol $\beta_5$) binding mode can be formulated using the Epstein combinatorial theory for large ligand

**TABLE II**

| Parameter | Substrate C | Substrate D | Substrate E |
|-----------|-------------|-------------|-------------|
| $K_{TP}$ (M$^{-1}$) | $(4 \pm 2) \times 10^7$ | $(2 \pm 0.5) \times 10^6$ | $(2 \pm 0.5) \times 10^6$ |
| $K_{DS}$ (M$^{-1}$) | $(2 \pm 0.5) \times 10^6$ | $(5 \pm 2) \times 10^7$ | $(7 \pm 3) \times 10^7$ |
| $K_S$ (M$^{-1}$) | $(8 \pm 3) \times 10^5$ | $(7 \pm 2) \times 10^5$ | $(9 \pm 2) \times 10^5$ |
| $\Delta F_{TP}$ | 1.9 $\pm$ 0.1 | 1.1 $\pm$ 0.1 | 1.5 $\pm$ 0.1 |
| $\Delta F_{DS}$ | 0.6 $\pm$ 0.1 | 0.6 $\pm$ 0.1 | 0.6 $\pm$ 0.1 |
| $\Delta F_S$ | 1.3 $\pm$ 0.1 | 1.2 $\pm$ 0.1 | 0.94 $\pm$ 0.06 |
| $\Delta F_{max}$ | 4.4 $\pm$ 0.2 | 4.1 $\pm$ 0.2 | 3.4 $\pm$ 0.2 |
| Maximum stoichiometry | 4.2 $\pm$ 0.3 | 4.3 $\pm$ 0.3 | 4.3 $\pm$ 0.3 |

Errors are standard deviations determined using three to four independent titration experiments.
binding to a finite, one-dimensional lattice (20, 24, 39). Thus, the complete partition function of the rat pol β-template-primer system, \( Z_{TP} \), is then as shown,

\[
Z_{TP} = Z_1 + \sum_{k=1}^{g} \sum_{j=0}^{k-1} S_{D}(k,j)(K_5P_5)^{5j} \omega^j
+ \sum_{k=1}^{g} \sum_{j=0}^{k-1} S_{DS}(k,j)(K_5P_5)^{5j} \omega^j
\]

where \( K_5 \) is the intrinsic binding constant of rat pol β in the (pol \( 5 \)) binding mode, \( \omega \) is the parameter characterizing cooperative interactions between the bound pol β molecules in this binding mode, \( g \) is the maximum number of rat pol β molecules which can bind to the ssDNA extension, at saturation (in our case \( g = 3 \)), \( k \) is the number of protein molecules bound at a given \( P_5 \), and \( j \) is the number of cooperative contacts between the \( k \) bound pol β molecules in a particular configuration on the lattice (20, 24, 39). The factor \( S_{D}(k,j) \) is the number of distinct ways that the \( k \) ligands bind to a lattice, with \( j \) cooperative contacts, and is defined by (39) in Equation 9.

\[
S_{D}(k,j) = \frac{\left[ (M - mh + 1)!(k - 1)! \right]}{\left[ (M - mh - k + j + 1)!(k - j - 1)! \right]} \]  

(Eq. 8)

The total degree of binding, \( \Sigma \nu_i \), is then defined as Equation 10.

\[
\Sigma \nu_i = Z_{TP}
\]

(Eq. 10)

The observed fluorescence increase, \( \Delta F \), is described by,

\[
\Delta F_5 = \Delta F_5^{b} + \Delta F_5^{c} + \Delta F_5^{d} + \Delta F_5^{e}
\]

(Eq. 9)

where \( \Delta F_5^{b} \) is the molar fluorescence increase accompanying the binding of the polymerase to the dsDNA part of the substrate; \( \Delta F_5^{c} \) and \( \Delta F_5^{d} \) are molar fluorescence increases accompanying the formation of the template-primer complex and the (pol \( 5 \)) binding mode, respectively. It should be pointed out that the quantity, \( \Delta F_5^{d} \), is averaged over all 33 possible complexes of the enzyme with the ssDNA extension in the (pol \( 5 \)) binding mode. Because the number of formed possible complexes is very large, such averaging does not introduce a significant error in the determination of the interaction parameters (20, 24).

There are four interaction parameters, \( K_{TP} \), \( K_{DS} \), \( K_5 \), and \( \omega \) and three fluorescence changes accompanying the formations of different complexes \( \Delta F_{TP} \), \( \Delta F_{DS} \), and \( \Delta F_5 \), which describe the binding process. Because the substrate has the same dsDNA part as the previously examined DNA substrates with the ssDNA extension of 5 nucleotide residues in length (Fig. 1), we assign \( K_{DS} = (2 \pm 0.5) \times 10^9 \text{ M}^{-1} \) and \( \Delta F_{DS} = 0.6 \pm 0.05 \) to this binding process. The remaining five parameters are still a formidable number of parameters, which precludes any attempt to obtain these quantities in a single fitting procedure.

The determination of all interaction and spectroscopic parameters of this very complex binding system can be achieved by applying the following strategy. Inspection of the isotherms in Fig. 3, a and b, shows that, due to its much higher macroscopic affinity, the formation of the template-primer complex is significantly separated from the binding to the dsDNA and the (pol \( 5 \)) binding mode on the protein concentration scale. This separation of the binding processes allows us to independently estimate \( \Delta F_{TP} \) as the slope, \( \Delta F_{TP} = \Delta \lambda F/\Delta \Sigma v_i \), of the initial part of the plot in Fig. 3b which provides \( \Delta F_{TP} = 1.9 \pm 0.1 \). Also, because initially the association of the enzyme with the DNA is completely dominated by the formation of the template-primer complex, we can determine the value of \( K_{TP} \) from the initial dependence of \( \Sigma v_i \) as a function of the protein concentration (plot not shown). The obtained estimate is \( K_{TP} = (4 \pm 2) \times 10^7 \text{ M}^{-1} \). The estimate of the average value of \( \Delta F_5 \) is based on the fact that the final complex, at saturation, must contain one rat pol β bound to the dsDNA and three pol β molecules associated with the ssDNA extension in the (pol \( 5 \)) binding mode. Therefore, the value of \( \Delta F_{max} = \Delta F_{DS} + 3 \Delta F_5 \) provides \( \Delta F_5 = 1.3 \pm 0.1 \). Finally, there are two remaining parameters that must be determined, \( K_5 \) and \( \omega \).

The solid lines in Fig. 3a are nonlinear least-square fits of the experimental isotherms to Equation 11 which provide \( K_5 = (8 \pm 3) \times 10^7 \text{ M}^{-1} \) and \( \omega = 5 \pm 2 \). It should be pointed out that the value of \( K_5 \) is similar to the \( K_5 \) obtained for the DNA substrate with the 3′ ssDNA extension at the 3′ end of the template where the pol β can only bind in the (pol \( 5 \)) binding mode (Table I). Such simplicity reflects the fact that both binding constants characterize a similar protein-nucleic acid binding process, i.e. the formation of the (pol \( 5 \)) binding mode. The value of \( \omega \) is in excellent agreement with previous studies which showed that the cooperative interactions between the rat pol β molecules bound in the (pol \( 5 \)) binding mode to the ssDNA extension at the 3′ end of the template (Fig. 1, substrate B). Notice, \( \omega \) is in the low range of 20 (20). The binding isotherms in Fig. 3a were also analyzed by letting four parameters float to within 20% of the determined values, e.g. \( K_{TP} \), \( \Delta F_{TP} \), \( \Delta F_{DS} \), and \( K_{DS} \), and treating \( K_5 \), \( \Delta F_5 \), and \( \omega \) as free fitting parameters. Within experimental accuracy, this procedure returns the same values of binding and spectroscopic parameters as the physically more intuitive approach described above (data not shown).

Further examination of the effect of the dsDNA on the binding of rat pol β to the template-primer DNA in the (pol \( 5 \)) and (pol \( 5 \)) binding modes has been performed using the DNA substrate having the ssDNA extension 15 nucleotide residues in length at the 3′ end of the template (Fig. 1; substrate D). Notice, with this substrate the polymerase cannot form the template-primer complex. The enzyme can bind the ssDNA extension only in either the (pol \( 5 \)) or the (pol \( 5 \)) binding mode.

Fluorescence titrations of the DNA substrate, with rat pol β at two different nucleic acid concentrations, in buffer C (pH 7.0, 10 °C) containing 100 mm NaCl, are shown in Fig. 3c. The relative increase of the nucleic acid fluorescence reaches the value of 4.1 ± 0.2 at saturation. The dependence of the relative fluorescence increase, as a function of the average degree of binding, \( \Sigma \nu_i \), is shown in Fig. 3d. There are two binding phases with different affinities. In the high affinity phase, the degree of binding reaches the value of 1 ± 0.2, indicating binding of a single molecule of the enzyme. Extrapolation of \( \Sigma \nu_i \) in the low affinity binding phase to the maximum fluorescence increase, \( \Delta F_{max} = 4.1 \pm 0.2 \), provides a value of \( \Sigma \nu_i = 4.3 \pm 0.3 \). Thus, at maximum saturation, four pol β molecules can bind to the DNA substrate with the ssDNA extension located at the 3′ end of the template. The number of bound pol β molecules is the same as observed for the DNA substrate with the ssDNA extension at the 5′ end of the template (Fig. 3b; Table II).
The DNA substrate with the 5′ssDNA extension (Fig. 3, A). The only difference is the nature of the high affinity complex of rat pol β final maximum stoichiometry of four rat pol β molecules associated with the ssDNA extension. At the same time, independent association of one polymerase molecule with the primer, on rat pol β, binding mode to the dsDNA and three pol β molecules bound in the (pol β)₅ binding mode. Thus, the value of ΔF_DS provides ΔF₀ = 1.2 ± 0.1. Finally, there are only two remaining parameters that must be determined, Kᵣ and ω.

The solid lines in Fig. 3c are nonlinear least-square fits, using Equations 7–11 which provide Kᵣ = (5 ± 2) × 10⁵ M⁻¹ and ω = 4 ± 2, respectively (Table II). Thus, the formation of the (pol β)₅ binding mode is characterized by virtually the same interaction parameters as that of the DNA substrate with the 5′ssDNA extension. Notice, the obtained value, Kᵣ₁₆ = (5 ± 2) × 10⁵ M⁻¹, is by factors of ~125 and ~250 higher than the intrinsic binding constant of the (pol β)₅ binding mode formed with the polymer and oligomer ssDNAs, indicating that the presence of the dsDNA affects the affinity of the (pol β)₅ binding mode to an even greater extent than that previously determined for the (pol β)₅ binding mode (Tables I and II) (20).

Such a significantly higher value of Kᵣ₁₆ strongly indicates that, when the enzyme is bound in the (pol β)₅ binding mode to the considered substrate, the small 8-kDa domain interacts with the dsDNA providing an additional contribution to the free energy of binding (see “Discussion”).

**Binding of Rat Pol β to the Gapped DNA Substrates**—The experiments and analyses described above focused on an effect of the presence of the primer, or the dsDNA downstream from the primer, on rat pol β interactions with the ssDNA extension in the (pol β)₁₆ or (pol β)₅, binding mode. In the base-excision repair processes, one of the physiological substrates of rat pol β is a gapped DNA which has a stretch of ssDNA embedded between the primer and the dsDNA, downstream from the primer (7, 21, 22).

The gapped DNA substrates, used to examine interactions with rat pol β, are depicted in Fig. 4. All DNA substrates contain two dsDNA parts each having 10 bp. The primary structure of the dsDNA parts is identical in all gapped DNAs and is the same as analogous dsDNA fragments in the previously analyzed DNA substrates (Figs. 1; substrates A, B, C, and D). The dsDNA parts are separated by a ssDNA gap having five nucleotide residues in length (Fig. 4; substrates E and F).

The obtained pattern of binding and the high stoichiometry can easily be understood as a consequence of the existence of the two ssDNA enzyme binding modes (see above; Ref. 20). At low enzyme concentrations, a single molecule of rat pol β associates with the ssDNA. However, with the ssDNA extension located at the 3′ end of the template, the enzyme cannot form a template-primer complex. In other words, only binding in the (pol β)₁₆ binding mode can occur at a low enzyme concentration, which corresponds to the high affinity step (Fig. 3d). As the polymerase concentration increases, the (pol β)₁₆ complex is replaced by the (pol β)₅ binding mode with three enzyme molecules associated with the ssDNA extension. At the same time, independent association of one polymerase molecule with the dsDNA part of the DNA substrate occurs providing the final maximum stoichiometry of four rat pol β molecules bound to the DNA.

The complexity of this binding system is similar to the complexity of the previously analyzed system of rat pol β binding to the DNA substrate with the 5′ssDNA extension (Fig. 3, a and b). The only difference is the nature of the high affinity complex. Instead of the template-primer complex, characterized by the binding constant, Kᵥ, the (pol β)₁₆ binding mode is formed with the affinity characterized by the binding constant, K₁₆. Therefore, the same model, as described by Equations 7–11, applies to the isotherms in Fig. 3c, with Kᵥ and ΔFᵥ replaced by K₁₆ and ΔF₁₆, respectively. Also, due to the large number of interaction and spectroscopic parameters, the extraction of these parameters follows the same strategy as outlined above.

The binding of rat pol β in the (pol β)₁₆ binding mode is significantly separated from the binding to the dsDNA and the (pol β)₅ binding mode, with respect to the protein concentration. This separation allows us to independently determine F₁₆ as the slope, ΔF₁₆ = ΔΔF/δΣvᵣ, of the initial part of the plot in Fig. 3d which provides ΔF₁₆ = 1.1 ± 0.1. The value of K₁₆ can be determined from the dependence of Σvᵣ as a function of the protein concentration (plot not shown) which provides K₁₆ = (5 ± 2) × 10⁷ M⁻¹. The dsDNA part of the substrate is the same as other studied substrates (Table I). Therefore, association of rat pol β with the dsDNA-binding site is described by the same parameters, K_DS = 2 × 10⁶ M⁻¹ and ΔF_DS = 0.6. The final complex, at saturation, contains one pol β molecule bound to the dsDNA and three pol β molecules bound in the (pol β)₅ binding mode. Thus, the value of ΔF_DS = ΔF_DS + 3ΔF₀ provides ΔF₀ = 1.2 ± 0.1. Finally, there are only two remaining parameters that must be determined, Kᵣ and ω.

**Fluorescence titrations of the DNA substrate**, having a ssDNA gap of five nucleotide residues in length (Fig. 4; substrate A), with rat pol β at two different nucleic acid concentrations, in buffer C (pH 7.0, 10 °C), containing 100 mM NaCl, are shown in Fig. 5a. The relative nucleic acid fluorescence increase reaches the value of 7.2 ± 0.3 at saturation. Fig. 5b shows the dependence of the observed relative fluorescence increase as a function of the average degree of binding, Σvᵣ, of the enzyme. The plot shows nonlinear behavior indicating two binding phases. In the high affinity phase, the degree of binding reaches the value of 1 ± 0.2, thus, a single molecule of the polymerase binds in this phase. However, the relative fluorescence increase, as determined from the initial slope, δΔF/δΣvᵣ,
of the plot is only 1.8 ± 0.1, very similar to the values obtained for the DNA substrates having ssDNA extensions with 15 nucleotide residues (Table I and II). Most of the fluorescence increase comes from the additional binding of pol β molecules to the DNA. Extrapolation of the low affinity phase to the maximum fluorescence increase, \( \Delta F_{\text{max}} = 7.2 \pm 0.3 \) provides a value of \( \Sigma v_1 = 3.3 \pm 0.3 \). Thus, the data indicate that, at saturation, three rat pol β molecules bind to the considered gapped DNA substrate.

The rat pol β affinity for the dsDNA is much lower than the affinity observed for the first binding phase (Tables I and II), therefore, the high affinity phase must correspond to the formation of a complex that includes the ssDNA gap. The low affinity phase must then correspond to the association of the remaining two pol β molecules with the two dsDNA parts of the substrate. Binding to the dsDNA, downstream from the primer, is most probably not affected by the enzyme molecule associated with the gap. However, this may not be the case with the binding to the dsDNA part at the primer location. Due to the limited size of the gap, the enzyme bound to the gap may be forced to invade the duplex DNA to a larger extent than in the previously examined substrates with ssDNA extensions where the large 31-kDa domain partially engages the ssDNA in the complex (see “Discussion”). Thus, in general, two dsDNA parts of the gapped DNA substrate may not be equivalent. In the simplest approach, the association of rat pol β with the gapped DNA can be treated as ligand binding to three distinct binding sites. The total partition function, \( Z_G \), of the system is defined by,

\[
Z_G = 1 + (K_{DS1} + K_{DS2} + K_0)F_p + (K_{DS1}K_{DS2} + K_{DS1}K_G + K_{DS2}K_G)F_p^2 + (K_{DS1}K_{DS2}K_G)F_p^3 \tag{Eq. 12}
\]

where \( K_G \) is the binding constant characterizing the association with the ssDNA gap, \( K_{DS1} \) and \( K_{DS2} \) are the binding constants characterizing the association with the dsDNA downstream from the primer and at the primer location, respectively. The degree of binding, \( \Sigma v_1 \), is then defined as,

\[
\Sigma v_1 = \frac{1}{(K_{DS1} + K_{DS2} + K_0)F_p + 2(K_{DS1}K_{DS2} + K_{DS1}K_G + K_{DS2}K_G)F_p^2 + 3(K_{DS1}K_{DS2}K_G)F_p^3} \tag{Eq. 13}
\]

The observed relative fluorescence change, \( \Delta F \), is described by,

\[
\Delta F = \frac{F \cdot F_p + F \cdot F_p^2 + F \cdot F_p^3}{Z_G} \tag{Eq. 14}
\]

where

\[
F_1 = (\Delta F_{\text{DS1}} + \Delta F_{\text{DS2}} + \Delta F_{\text{DS1}} + \Delta F_{\text{DS2}})K_{DS1}K_{DS2} + (\Delta F_{\text{DS1}} + \Delta F_{\text{DS2}})K_{DS1}K_G + (\Delta F_{\text{DS2}} + \Delta F_{\text{DS1}})K_{DS2}K_G + \Delta F_GK_{DS1}K_{DS2}K_G \tag{Eq. 15}
\]

\[
F_2 = [\Delta F_{\text{DS1}} + \Delta F_{\text{DS2}}]K_{DS1}K_{DS2} + (\Delta F_{\text{DS1}} + \Delta F_{\text{DS2}})K_{DS1}K_G + (\Delta F_{\text{DS2}} + \Delta F_{\text{DS1}})K_{DS2}K_G + \Delta F_GK_{DS1}K_{DS2}K_G \tag{Eq. 16}
\]

\[
F_3 = (\Delta F_{\text{DS1}} + \Delta F_{\text{DS2}} + \Delta F_G)K_{DS1}K_{DS2}K_G \tag{Eq. 17}
\]

There are six independent parameters in Equations 12–17. As we pointed out above, such a large number of parameters precludes their determination in a single, fitting procedure. The approach to extract them is similar to the one already described for the other DNA substrates (see above). Much higher affinity of the nucleic acid to the gap region than the dsDNA parts of the nucleic acid allows us to determine \( \Delta F_G \) as the slope, \( \Delta F_G = \Delta F / \Delta \Sigma v_1 \), of the initial part of the plot in Fig. 5b, which provides \( \Delta F_G = 1.8 \pm 0.1 \). The value of \( K_G \) can be determined from the analysis of the dependence of \( \Sigma v_1 \), as a function of the protein concentration in the low protein concentration range (plot not shown), which provides \( K_G = (5 \pm 2) \times 10^7 \text{ M}^{-1} \). Association of rat pol β with the dsDNA, downstream from the primer, is characterized by the same parameters, \( K_{DS1} = (2 \pm 0.5) \times 10^8 \text{ M}^{-1} \) and \( \Delta F_{DS1} = 0.6 \pm 0.1 \), as determined for this binding site for other studied substrates (Tables I and II). At saturation, the complex contains one rat pol β bound to the gap and two enzyme molecules associated with each of the dsDNA parts of the substrate. Therefore, the value of \( \Delta F_{\text{max}} = \Delta F_{\text{DS1}} + \Delta F_{\text{DS2}} + \Delta F_G \) which provides \( \Delta F_{\text{DS1}} = 5 \pm 0.1 \). Thus, there is only one remaining parameter that has to be determined, \( K_{DS1} \).

The solid lines in Fig. 5a are nonlinear least-square fits of the experimental isotherms to Equations 12–17 with \( K_{DS1} = (3 \pm 1) \times 10^7 \text{ M}^{-1} \). The theoretical curves provide an excellent description of the experimental titration curves. Alternatively, knowing \( \Delta F_G, \Delta F_{DS1}, \Delta F_{DS2}, \) and \( K_{DS1} \), or letting these parameters float to within 20% of the estimated values, one can fit the experimental curves with two parameters \( K_G \) and \( K_{DS1} \) (data not shown). Within experimental accuracy, these procedures return very similar values of all binding parameters as the more intuitive approach described above.

The obtained value of \( K_G \) for the ssDNA gap of 5 nucleotide residues is a factor of ~2000 higher than the intrinsic binding constant, \( K = 2 \times 10^4 \text{ M}^{-1} \), characterizing the formations of only the (pol β)_2 binding mode formed exclusively on the ssDNA oligomer (20). The determined affinity of the enzyme for the gap is by factors of ~160 and ~100 higher than the affinities for the DNA substrates with the ssDNA extension capable of accommodating the enzyme only in the (pol β), binding mode (Tables I). Also, the value of \( K_G \) is by a factor of ~25 higher than the value of \( K_{DS1} \) characterizing the independent binding to the dsDNA (Tables I and III). It is evident that such a large difference in affinity provides the enzyme with a significant preference for the gapped DNA, as compared with the ss and dsDNAs.

Examination of rat pol β binding to gapped DNA substrates, having ssDNA gaps of different sizes (Fig. 4), addresses the very important question: how is the high affinity and stoichiometry for the enzyme-gap complex affected by the size of the ssDNA gap? The smallest possible ssDNA gap is 1 nucleotide in length. Fluorescence titrations of the DNA substrate having only 1 nucleotide residue in the ssDNA gap, with rat pol β, at two different nucleic acid concentrations, in buffer C (pH 7.0, 10 °C), containing 100 mM NaCl, are shown in Fig. 5c. The relative nucleic acid fluorescence increase reaches the value of \( 2.1 \pm 0.2 \) at saturation, which is much lower than the maximum fluorescence increase observed for the gapped DNA substrate with 5 nucleotide residues in the ssDNA gap (Fig. 5a; see “Discussion”). Fig. 5d shows the dependence of the observed relative fluorescence increase as a function of the average degree of binding, \( \Sigma v_1 \), of the enzyme on the substrate. There are clearly two binding phases. In the high affinity phase, the degree of binding reaches the value of \( 1 \pm 0.2 \), indicating the binding of a single molecule of the polymerase. The relative fluorescence increase accompanying the binding in this step is \( \Delta F_G / \Delta \Sigma v_1 = 0.9 \pm 0.1 \). Extrapolation of the low affinity phase to the maximum fluorescence increase, \( \Delta F_{\text{max}} = 2.1 \pm 0.2 \) provides a value of \( \Sigma v_1 = 3.1 \pm 0.3 \). Thus, despite the fact that the considered substrate has only a single nucleotide residue in the ssDNA gap, at maximum saturation three rat pol β molecules bind to the substrate.

Titration curves in Fig. 5c have been analyzed as already described for the gapped DNA substrate with 5 nucleotide residues in the ssDNA gap, using the three-site binding model as described by Equations 12–17. The solid lines in Fig. 5c are nonlinear least-square fit of the experimental isotherms with \( K_G = (2 \pm 1) \times 10^7 \text{ M}^{-1} \), \( \Delta F_G = 0.9 \pm 0.1 \), \( K_{DS1} = (2 \pm 0.5) \times 10^8 \text{ M}^{-1} \),...
Fig. 5. a, fluorescence titrations (λex = 325 nm, λem = 410 nm) of the gapped DNA substrate (Fig. 4; substrate A), with the ssDNA gap having 5 nucleotide residues, with rat pol β in buffer C (pH 7.0, 10 °C), containing 100 mM NaCl, at 2 different concentrations of the nucleic acid: ■ 4.5 × 10−7 M, □ 1.5 × 10−7 M. The solid lines are nonlinear least-square fits of the fluorescence titration curves according to the three binding-site model. The lines are plotted using a single set of parameters: KG = 5 × 105 M−1, KDSD1 = 2 × 108 M−1, KDSD2 = 3 × 105 M−1, ΔF0 = 1.8, ΔFDS2 = 0.6, and ΔFDS = 5 (Table III; details in text). b, the dependence of the relative fluorescence increase, ΔF, upon the degree of binding, Σνj, of the rat pol β-gapped DNA complex. The values of the degree of binding have been determined using the quantitative method described under “Experimental Procedures.” The solid lines are the limiting slopes of the two binding phases. The dashed line is an extrapolation of the degree of binding to the maximum value of the observed fluorescence increase ΔFmax = 7.2 ± 0.2 that provides the maximum stoichiometry of 3.3 ± 0.3 of the rat pol β-gapped DNA complex. c, fluorescence titrations of the gapped DNA substrate (Fig. 4; substrate E) having the ssDNA gap with 1 nucleotide residue, with rat pol β in buffer C (pH 7.0, 10 °C), containing 100 mM NaCl, at 2 different concentrations of the nucleic acid: ■ 8.8 × 10−7 M, □ 2.22 × 10−6 M. The solid lines are nonlinear least-square fits of the fluorescence titration curves according to the three binding-site model (Table III; details in text). d, the dependence of the relative fluorescence increase, ΔF, upon the degree of binding, Σνj, of the rat pol β-gapped DNA complex (Fig. 4; substrate E). The dashed line is an extrapolation of the degree of binding to the maximum value of the observed fluorescence increase ΔFmax = 2.1 ± 0.2 that provides the maximum stoichiometry of 3.1 ± 0.3.

106 M−1, ΔFDS2 = 0.6 ± 0.1, KDSD2 = (2.5 ± 0.8) × 108 M−1, and ΔFDS = 0.6 ± 0.1. The interaction and spectroscopic parameters for all studied gapped DNA substrates are included in Table III. Thus, the size of the ssDNA gap does not affect the enzyme affinity for the ssDNA gap, as reflected in virtually the same values of KG for all examined substrates. On the other hand, there are differences between different gapped DNAs, with respect to the values of the maximum fluorescence increase, ΔFmax, and spectroscopic parameters, ΔF0 and ΔFDS. These differences suggest that, despite very similar affinities, the ssDNA in formed complexes are different (see “Discussion”).

Effect of the 5’ Terminal Phosphate Downstream from the Primer on the Rat Pol β Association with the Template-Primer and Gapped-DNA Substrates—Steady-state kinetic studies of rat pol β activities on the template-primer and gapped DNA substrates clearly indicate that the presence of the 5’ terminal phosphate group, downstream from the primer, plays an important role in DNA substrate recognition and catalysis (21–23). However, direct thermodynamic analysis of the effect of the 5’ terminal phosphate on the binding of rat pol β to DNA substrates has never been addressed before. To elucidate the role of the 5’ terminal phosphate group in the energetics of the polymerase interactions with the DNA, we first examined the interaction of rat pol β with the DNA substrate having a 5’ terminal phosphate on the oligonucleotide forming the dsDNA at the 5’ end of the template strand (Fig. 1; substrate B). The relative maximum nucleic acid fluorescence increase reaches the value of ΔFmax = 3.4 ± 0.2, at saturation, which is significantly lower than ΔFmax = 4.4 ± 0.2 obtained for the DNA substrate without the phosphate group (Table II). Thus, the data show that the presence of the 5’ terminal phosphate affects the extent of the structural changes in the ssDNA induced by the enzyme in the complex. At this point, it should be mentioned that in the presence of Mg2+, pol β catalyzes a slow release of the 5’ terminal deoxyribose phosphate (40). Therefore, we also examined the binding of the enzyme to the considered DNA substrate and the gapped DNA discussed below, in the absence of MgCl2 (data not shown). Both sets of data, in the presence and absence of Mg2+, give, within experimental accuracy, very similar results. Thus, in our solution conditions (low Mg2+ and low temperature) the catalysis is very slow, on
The theoretical lines are plotted using a single set of parameters.

The analysis of the titration curves in Fig. 6a has been performed in the same way as described for the DNA substrate having the ssDNA extension with 15 nucleotide residues at the 3′ end of the template strand (Figs. 3, c and d). The solid lines in Fig. 6a are nonlinear least-square fits of the experimental isotherms with \( K_{16} = (7 \pm 3) \times 10^4 \) M\(^{-1} \), \( K_{DS} = (2 \pm 0.5) \times 10^6 \) M\(^{-1} \), \( K_{FDS} = 0.6 \pm 0.1 \), \( K_5 = (9 \pm 2) \times 10^5 \) M\(^{-1} \), \( n = 3 \pm 1 \), and \( k_F = 0.94 \pm 0.06 \) (Table II). Thus, the presence of the 5′ terminal phosphate has little effect on the values of all spectroscopic and interaction parameters, as compared with the same DNA substrate without the phosphate group (Fig. 1; Table II).

Analogous fluorescence titrations have been performed using the DNA substrate with the ssDNA gap having 5 nucleotide residues and the 5′ terminal phosphate (Fig. 4; substrate F), with rat pol β (data not shown). In the high affinity phase, \( \Sigma v_i \) reaches the value of \( 1 \pm 0.2 \), indicating binding of a single enzyme molecule. Extrapolation of \( \Sigma v_i \) to the maximum fluorescence increase, \( \Delta F_{\text{max}} = 6.7 \pm 0.2 \), provides a value of \( \Sigma v_i = 3.2 \pm 0.3 \), indicating that the maximum stoichiometry of three pol β molecules bound to the DNA is not affected by the presence of the 5′ terminal phosphate. The relative fluorescence increase reaches the value of 6.7 ± 0.2, at saturation, which is similar to the same parameter obtained for the DNA substrate without the phosphate group (Table III). However, the slope, \( \Delta F = \Delta F_0 / (\Sigma v_i) = \Delta F_{16} \), of the initial part of the titration curve provides \( \Delta F_0 = 3 \pm 0.1 \). This value is significantly higher than the \( \Delta F_0 = 1.8 \pm 0.1 \) obtained for the DNA substrate without the terminal phosphate (Table III). Because \( \Delta F_0 \) characterizes the conformational state of the ssDNA of the gap, in the complex with the enzyme molecule directly bound to the gap, such a large value of \( \Delta F_0 \) indicates that the presence of the 5′ terminal phosphate strongly affects the conformational state of the ssDNA in the complex with the polymerase (see “Discussion”).

The dependence of the observed \( \Delta F \) as a function of the average degree of binding, \( \Sigma v_i \), of rat pol β on DNA, is shown in Fig. 6b. In the high affinity phase, the degree of binding reaches the value of \( 1 \pm 0.2 \), indicating binding of a single molecule of the enzyme. Extrapolation of \( \Sigma v_i \) to the maximum fluorescence increase, \( \Delta F_{\text{max}} = 3.4 \pm 0.2 \), provides a value of \( \Sigma v_i = 4.3 \pm 0.3 \). Clearly, the presence of the 5′ terminal phosphate does not affect the stoichiometry of the complex. The slope, \( \Delta F = \Delta F_0 / (\Sigma v_i) = \Delta F_{16} \), of the initial part of the plot in Fig. 6b provides \( \Delta F_{16} = 1.5 \pm 0.1 \). This value is slightly higher than the one obtained for the DNA substrate without the terminal phosphate (Table II), indicating different ssDNA structures in both complexes.

The time scale of the titration experiments and does not affect the studied equilibrium.

The dependence of the observed \( \Delta F \) as a function of the average degree of binding, \( \Sigma v_i \), of rat pol β on DNA, is shown in Fig. 6b. In the high affinity phase, the degree of binding reaches the value of \( 1 \pm 0.2 \), indicating binding of a single molecule of the enzyme. Extrapolation of \( \Sigma v_i \) to the maximum fluorescence increase, \( \Delta F_{\text{max}} = 3.4 \pm 0.2 \), provides a value of \( \Sigma v_i = 4.3 \pm 0.3 \). Clearly, the presence of the 5′ terminal phosphate does not affect the stoichiometry of the complex. The slope, \( \Delta F = \Delta F_0 / (\Sigma v_i) = \Delta F_{16} \), of the initial part of the titration curve provides \( \Delta F_{16} = 1.5 \pm 0.1 \). This value is slightly higher than the one obtained for the DNA substrate without the terminal phosphate (Table II), indicating different ssDNA structures in both complexes.

The analysis of the titration curves in Fig. 6a has been performed in the same way as described for the DNA substrate having the ssDNA extension with 15 nucleotide residues at the 3′ end of the template strand (Figs. 3, c and d). The solid lines in Fig. 6a are nonlinear least-square fits of the experimental isotherms with \( K_{16} = (7 \pm 3) \times 10^4 \) M\(^{-1} \), \( K_{DS} = (2 \pm 0.5) \times 10^6 \) M\(^{-1} \), \( K_{FDS} = 0.6 \pm 0.1 \), \( K_5 = (9 \pm 2) \times 10^5 \) M\(^{-1} \), \( n = 3 \pm 1 \), and \( k_F = 0.94 \pm 0.06 \) (Table II). Thus, the presence of the 5′ terminal phosphate has little effect on the values of all spectroscopic and interaction parameters, as compared with the same DNA substrate without the phosphate group (Fig. 1; Table II).

Analogous fluorescence titrations have been performed using the DNA substrate with the ssDNA gap having 5 nucleotide residues and the 5′ terminal phosphate (Fig. 4; substrate F), with rat pol β (data not shown). In the high affinity phase, \( \Sigma v_i \) reaches the value of \( 1 \pm 0.2 \), indicating binding of a single enzyme molecule. Extrapolation of \( \Sigma v_i \) to the maximum fluorescence increase, \( \Delta F_{\text{max}} = 6.7 \pm 0.2 \), provides a value of \( \Sigma v_i = 3.2 \pm 0.3 \), indicating that the maximum stoichiometry of three pol β molecules bound to the DNA is not affected by the presence of the 5′ terminal phosphate. The relative fluorescence increase reaches the value of 6.7 ± 0.2, at saturation, which is similar to the same parameter obtained for the DNA substrate without the phosphate group (Table III). However, the slope, \( \Delta F = \Delta F_0 / (\Sigma v_i) = \Delta F_{16} \), of the initial part of the titration curve provides \( \Delta F_0 = 3 \pm 0.1 \). This value is significantly higher than the \( \Delta F_0 = 1.8 \pm 0.1 \) obtained for the DNA substrate without the terminal phosphate (Table III). Because \( \Delta F_0 \) characterizes the conformational state of the ssDNA of the gap, in the complex with the enzyme molecule directly bound to the gap, such a large value of \( \Delta F_0 \) indicates that the presence of the 5′ terminal phosphate strongly affects the conformational state of the ssDNA in the complex with the polymerase (see “Discussion”).

The nonlinear least-square fit of the experimental isotherms, using Equations 12–17 provide \( K_G = (5 \pm 2) \times 10^5 \) M\(^{-1} \), \( \Delta F_{G} = 3 \pm 0.1 \), \( K_{{DS}} = (2 \pm 0.5) \times 10^6 \) M\(^{-1} \), \( \Delta F_{DS} = 0.6 \pm 0.1 \), \( K_{{DS2}} = (2.1 \pm 0.8) \times 10^6 \) M\(^{-1} \), and \( \Delta F_{DS2} = 3.1 \pm 0.1 \). Thus, despite the structural differences, as expressed by the much larger \( \Delta F_G \) the presence of the 5′ terminal phosphate does not significantly affect the energetics of rat pol β binding to the gapped DNA (Table III).
DISCUSSION

Despite simplified catalytic activities, the recognition process of the template-primer and gapped DNA substrates by rat pol β is a complex process, as indicated by the direct thermodynamic studies described in this work. Such complex behavior was already evident in previous studies of the binding of the enzyme exclusively to the ssDNA (20). The polymerase binds the ssDNA using two different binding modes, which have a different number of occluded nucleotide residues in the protein-ssDNA complex. These findings were possible due to the application of the quantitative fluorescence titration technique that allowed us to determine the average degree of binding, Σν, of the protein-DNA complexes, without assumptions about the relationship between the observed signal used to monitor the binding and the stoichiometry of the studied complexes (32–35). Moreover, the approach allows us to extract spectroscopic parameters characterizing the structural changes of the nucleic acid accompanying the formation of the particular complexes.

The Stoichiometry of Rat Pol β-Template-Primer DNA Substrate Complexes Is Higher Than Predicted by the Crystal Structure of the Enzyme-DNA Complex—A stoichiometry of the ligand-macromolecule complex, at saturation, is a model-independent parameter. This quantity defines the maximum number of ligand molecules that can bind to the macromolecule. A surprising feature of the examined interactions between the template-primer DNA substrates and rat pol β is the high stoichiometry of the formed complexes at saturation. Thus, four rat pol β molecules are bound to the DNA when the ssDNA extension has a length of 15 nucleotides. Even with the template-primer substrates, which have a ssDNA extension with only 5 nucleotide residues (Fig. 1; substrates A and B), two enzyme molecules associate with the DNA. These stoichiometries, determined in solution, are very different from the reported crystal structure of the similar enzyme-template-primer complexes, although, more recent extensive, crystallographic studies revealed the existence of more than one pol β molecule bound to the template-primer DNA (16, 17).

The observed stoichiometries can be understood in the context of the known enzyme affinity for the dsDNA conformation and the existence of the two ssDNA binding modes (20). Moreover, the analysis of the formed complexes is facilitated by the direct thermodynamic studies described in this work, which polymerase has two distinct DNA-binding domains possessing different preferences for the two nucleic acid conformations (14–19). The site with the preference for the ssDNA is located on the small 8-kDa domain, while the site with the preference for the dsDNA is located on the large 31-kDa catalytic domain (14, 18, 19).

The (pol β)16 binding mode, in which the enzyme occludes 16 ± 2 nucleotide residues, is formed in the large excess of the ssDNA (20). In this mode, both domains of the enzyme are engaged in interactions with the nucleic acid. The transition to the (pol β)5 binding mode is induced by the increased protein concentration resulting from the increase of the protein binding density on the DNA. Therefore, when the availability of the ssDNA is decreasing, the protein, initially bound to 16 nucleotide residues, is forced to bind the ssDNA with a lower 5-nucleotide site-size, forming the (pol β)5 binding mode, with the 8-kDa domain predominantly interacting with the DNA (20). The low availability of the ssDNA is already built into the structure of the template-primer DNA substrate, having the 3′ ssDNA extension with only 5 nucleotides. The extension can only accept one enzyme molecule in the (pol β)5 binding mode. The second enzyme molecule must bind to the 10-bp long dsDNA part of the substrate using the dsDNA-binding site located on the large 31-kDa domain (16, 17).

At low protein concentrations the template-primer substrate, having the 3′ ssDNA extension with 15 nucleotide residues, can initially accept a single pol β molecule only in the (pol β)16 binding mode. However, as the protein concentration increases, this mode is being replaced by the (pol β)5 binding mode leading to three enzyme molecules bound to the ssDNA extension, at saturation. Binding of the single rat pol β molecule to the dsDNA part of the substrate results in 4 enzyme molecules associated with the DNA.

For the template-primer substrates having the ssDNA extensions at the 5′ end of the substrate, the situation is different (Fig. 1). At low protein concentrations, the extension with 15 nucleotide residues can accept the enzyme in the (pol β)16 or the (pol β)5 binding mode. However, because the ssDNA extension is located at the 5′ end of the substrate, the enzyme forms a template-primer complex instead of the (pol β)16 binding mode, where the 8-kDa domain binds the ssDNA of the template and the large 31-kDa domain is engaged in interactions with the ss-ds DNA junction (16). The formation of such a complex is reflected in the different interaction and spectroscopic parameters, as compared with the DNA substrate having the ssDNA extensions with 15 nucleotide residues at the 3′ end of the substrates where only the (pol β)16 binding mode can be formed (Table II) (20). Also, a lower fluorescence change and a much higher affinity characterizing the binding of the enzyme to the DNA substrate with the ssDNA extension having 5 nucleotide residues indicate that, instead of the (pol β)5 binding mode, the polymerase binds the ss-ds DNA junction of the DNA substrate using its 31-kDa domain (16, 20).

Notice the maximum stoichiometries are not affected by either the existence of the template-primer complex or the binding to the ss-ds DNA junction. Moreover, the binding constants of the second pol β molecule are unaffected by the template-primer complex, indicating that the 10-bp long dsDNA is fully available to the enzyme (Table I). Thus, these data also suggest that in the template-primer complex, or when bound to the ss-ds DNA junction, contrary to the crystal structure of the complex, the polymerase must encompass less than 7 bp of the dsDNA of the substrate (16, 17).

The 8-kDa Domain Interacts with the dsDNA, Downstream from the Primer, in Both the (pol β)5 and (pol β)16 Binding Modes—Experiments with DNA substrates having the ssDNA extension at the 3′ end of the substrate provide strong evidence that the 8-kDa domain of rat pol β is involved in interactions with the dsDNA located at the 5′ end of the substrate. The intrinsic binding constants for the formation of the (pol β)5 and (pol β)16 binding modes, exclusively with the ssDNA oligomer d(ApεεA)15, in the same solution conditions as applied in this work, are \( K_5 = (2 \pm 1) \times 10^4 \text{ M}^{-1} \) and \( K_{16} = (2 \pm 1) \times 10^5 \text{ M}^{-1} \), respectively (20). However, the values of the corresponding constants determined for the template-primer substrates having the 3′ ssDNA extension, where the enzyme can only form either the (pol β)5 or the (pol β)16 binding mode, are \( K_5 = (7 \pm 2) \times 10^5 \text{ M}^{-1} \) and \( K_{16} = (5 \pm 2) \times 10^6 \text{ M}^{-1} \), respectively (Table II). Thus, in the presence of the dsDNA, downstream from the primer, the affinities of both binding modes are increased by factors of ∼35 and ∼250, respectively. These results strongly indicate that in both binding modes the 8-kDa domain is engaged in interactions with the dsDNA of the DNA substrate. On the other hand, there is a significant difference between the effects of the dsDNA on the two binding modes. A stronger effect is observed for the (pol β)16 binding mode, suggesting that in this binding mode the 8-kDa domain has more flexibility to engage the dsDNA. This conclusion is supported by the observed lower fluorescence changes accompanying the formation of the (pol β)16 binding mode, indicating a more flexible...
structure at the interface of the protein-nucleic acid complex (20). Such flexibility is limited in the (pol $\beta$)$_2$ binding mode where the enzyme predominantly interacts with the ssDNA through the 8-kDa domain, leading to a much stronger immobilization of the DNA and the domain in the complex (20).

Energetics of the Rat Pol $\beta$-Template-Primer Complex—As we discussed above, when the ssDNA extension with 15 nucleotide residues is located at the 5’ end of the DNA substrate (Fig. 1; substrate C), rat pol $\beta$ can form a template-primer complex (16, 17). In this complex the 8-kDa domain of the enzyme interacts with the ssDNA of the template, while the catalytic 31-kDa domain is engaged in interactions with the ss-ds DNA junction (16). The binding constant characterizing the formation of the template-primer complex $K_{\text{TP}} = (4 \pm 2) \times 10^7 \text{ M}^{-1}$ is by a factor of $\sim$2000 higher than the intrinsic binding constant determined for the (pol $\beta$)$_1$ binding mode ($K_p = (2 \pm 1) \times 10^4 \text{ M}^{-1}$), where the enzyme exclusively interacts with the ssDNA using the 8-kDa domain, and by a factor of $\sim$10 higher than the binding constant $K_p = (4.6 \pm 0.6) \times 10^6 \text{ M}^{-1}$, characterizing the independent interactions of the polymerase with the ss-ds DNA junction, through the large 31-kDa domain (Tables I and II).

Comparison of these binding constants provides the first indication that the association of the enzyme with the template-primer DNA is not composed of the independent interactions of the two domains of the enzyme with two different conformations of the DNA substrate. The free energy change accompanying the formation of the template-primer complex is

$$\Delta G_{\text{TP}} = R T \ln K_{\text{TP}},$$

where $R$ is the gas constant and $T$ is the temperature in Kelvin degrees (41). Introducing the value of $K_{\text{TP}}$ provides $\Delta G_{\text{TP}} = -9.9 \pm 0.2 \text{ kcal/mol}$.

Analogous calculation for the (pol $\beta$)$_2$ binding mode and the ss-ds DNA junction result in $\Delta G_{\text{DS}} = -5.5 \pm 0.3$ and $\Delta G_{\text{DS}} = -8.6 \pm 0.2 \text{ kcal/mol}$, respectively. The absolute value of free energy of the template-primer complex formation is significantly lower than the absolute value of the sum of independent free energies of binding ($14.1 \pm 0.5 \text{ kcal/mol}$) exclusively in the (pol $\beta$)$_2$ binding mode and to the ss-ds DNA junction.

The free energy cost of the template-primer complex formation can be approximately obtained using a general approach introduced by Jencks (42). The free energy of simultaneous binding of the ssDNA and the ss-ds DNA junction, as parts of the template-primer DNA, can be defined as,

$$\Delta G_{\text{TP}} = \Delta G_1 + \Delta G_2 - \Delta G_0$$

(18)

where $\Delta G_0$ is the “connection” free energy change representing a gain or loss of free energy as a result of the simultaneous binding of the ss- and dsDNAs as structural parts of the larger substrate, the template-primer DNA. Introducing the values of $\Delta G_{\text{TP}}, \Delta G_0$, and $\Delta G_2$ into Equation 10 provides $\Delta G_0 = 4.2 \pm 0.7 \text{ kcal/mol}$.

Notice that the value of $\Delta G_0$ is positive, thus, the complex of the enzyme with the template-primer substrate has a significant excess of the free energy gained at the expense of the free energy of binding of the ssDNA and the ss-ds DNA junction of the substrate. At this point, it is not known whether or not the excess free energy is accumulated in the polymerase or the DNA or both. Elegant crystallographic studies indicate large conformational changes in both pol $\beta$ and the DNA substrate in the enzyme-DNA complex, as compared with the structures of both macromolecules in the absence of interactions (16). Such excess free energy can be released at different stages of enzyme action whenever interactions with one of the DNA conformations are weakened. For instance, the free energy release can play an important role in the functioning of the enzyme, particularly in the mechanical translocation, where the affinities of the enzyme domains for different conformations of the DNA substrate transiently change (1, 16, 17, 43). Because of similarities of the enzyme structure and its complex with the template-primer DNA among different polymerases, it is very possible that a similar pattern of free energy changes, i.e. accumulation of $\Delta G$ at the expense of the free energy of binding accompanying interactions of other DNA polymerases with a template-primer DNA.

The Size of the ssDNA Gap Has Little Effect on the Stoichiometry and Affinity of the Rat Pol $\beta$ Complexes with Different Gapped DNA Substrates—The maximum stoichiometry of the enzyme-DNA complex and the intrinsic affinities of the bound pol $\beta$ molecules, to various gapped DNA substrates examined in this work, are not affected by the diminishing size of the ssDNA gap (Table III). Thus, the same stoichiometry of the complex is preserved despite the fact that the largest and the smallest gaps differ by as much as 4 nucleotide residues that are within the site-size of $5 \pm 2$ of the (pol $\beta$)$_2$ binding mode (20). It is still very possible that the formation of the (pol $\beta$)$_2$ binding mode is a part of the gap recognition mechanism, particularly for the larger gaps. Whether or not the (pol $\beta$)$_2$ binding mode is a part of the recognition mechanism for large ssDNA gaps can only be resolved by kinetic methods. However, the obtained results indicate that the final equilibrium complex with the gap is very different from the (pol $\beta$)$_2$ binding mode.

Steady-state kinetic data have clearly shown that the polymerase can processively catalyze the DNA synthesis on all DNA substrates, analogous to the ones used in this work (21, 22). Thus, the active site of the enzyme, located on the 31-kDa catalytic domain, must always be placed close to the 3’ end of the primer oligomer (16). In other words, in all examined gapped DNA substrates, the 31-kDa domain interacts with the ss-ds DNA junction in the same orientation, with respect to the dsDNA. Therefore, what has to change, to preserve the same stoichiometry and the same intrinsic affinities of all three bound pol $\beta$ molecules as the size of the gap diminishes, is the orientation of the 8-kDa domain of the enzyme molecule bound to the gap, with respect to its large domain and the gap. The independence of the high intrinsic affinity of the enzyme molecule, strictly bound to the ssDNA gap, upon the size of the gap, indicates that in the complexes with different gapped DNAAs, the enzyme forms all crucial contacts with the DNA substrate. Studies of rat pol $\beta$ interactions with the template-primer DNA substrates, described above, clearly indicate that the small 8-kDa domain of the enzyme can engage in interactions with the dsDNA (see above). This result is of paramount importance because the dsDNA is the only nucleic acid conformation available to the 8-kDa domain in the complexes with small gaps. The unaffected affinity would reflect the fact that, independently of the size of the gap of the examined DNA substrates, both domains are interacting with the same conformation of the DNA. The 31-kDa domain is bound to the junction of the ss- and dsDNA, while the 8-kDa domain is bound to the dsDNA, downstream from the primer.

Model for the Gapped-DNA Substrate Recognition by Rat Pol $\beta$—The ssDNA affinity of the 8-kDa domain has been invoked as a major factor in the recognition of gapped-DNA substrates (22). Although, as we stated above, this may still be true, particularly for the larger gaps, the recognition of the small gaps cannot rely on such ssDNA affinity because there is not enough ssDNA available for binding (20). We propose the following plausible mechanism of the small ssDNA gap recognition by rat pol $\beta$ on the basis of the results and analyses obtained in this work.

Rat pol $\beta$ binds the dsDNA, as well as the ss-ds DNA junction of the substrate, using its large catalytic 31-kDa domain. Bind-
to engage in interactions with the dsDNA. The polymerase remains in its unchanged conformation with the 8-kDa domain at a significant distance from the nucleic acid, and not able to bind the dsDNA conformation downstream from the primer. These additional interactions lead to the significantly amplified affinity for the gapped DNA substrate. Binding exclusively to the dsDNA (A) does not induce the conformational transition of the polymerase. As a result, the enzyme interacts with the nucleic acid, using only the affinity of its dsDNA-binding site located on the large 31-kDa domain. The polymerase remains in its unchanged conformation with the 8-kDa domain at a significant distance from the nucleic acid, and not able to engage in interactions with the dsDNA.

The enzyme binds both substrates using its large 31-kDa domain. However, in the complex with the gapped DNA, interactions between the 31-kDa domain (gray area) and the ss-ds DNA junction of the DNA substrate induce a specific allosteric transition of the enzyme. As a result, the small 8-kDa domain (black area), which has an intrinsic affinity for the dsDNA, is at a shorter distance from the DNA and binds the dsDNA conformation downstream from the primer. These interactions lead to the significantly increased affinity for the gapped DNA. As a result, the small 8-kDa domain (black area), which has an intrinsic affinity for the dsDNA, is at a shorter distance from the DNA and binds the dsDNA conformation downstream from the primer. As a result, the affinity of the enzyme for the DNA substrate with the 3' terminal phosphate downstream from the primer is 3 ± 0.1, as compared with the $\Delta F_G = 1.8 \pm 0.1$ determined in the absence of the PO$_4$ group (Table III). Because at the applied excitation wavelength ($\lambda_{ex} = 325$ nm), only ethenoadenosine is excited, the observed increase results from an increase of the quantum yield of the nucleic acid in the complex with the polymerase. The fluorescence of eA is dramatically quenched (8–12-fold) in etheno-oligomers and polymers as compared with the free AMP (44, 45). A dynamic model, in which the motion of eA leads to quenching via intramolecular collision, has been proposed as a predominant mechanism of the observed strong quenching (44). Thus, the fluorescence of eA in etheno-derivatives of the polymer and oligomer nucleic acids is predominantly affected by the mobility and separation of the bases and not by the polarity of the environment. Therefore, a large increase of the $\Delta F_G$ value indicates that, in the presence of the 5' terminal phosphate group, the ssDNA of the gap assumes a much more rigid conformation with significantly increased distances between the bases.

It is rather certain that recognition and DNA synthesis on the template-primer, or gapped DNA substrates, are complex, multistep processes that must include binding steps as well as chemical catalysis. In the context of the previous steady-state studies, our results indicate that the presence of the 5' terminal phosphate group has an effect on the functioning of the enzyme, not through increasing the ground-state affinity, but by affecting some of the subsequent chemical steps. Large differences between the ssDNA conformations in the gap, in the absence and presence of the terminal phosphate, provide the first structural clue as to how this can happen. The increased 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.

**Acknowledgment**—We thank Gloria Drennan Davis for help in preparing the manuscript.

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J. Biol. Chem. 2001, 276:16123-16136.
doi: 10.1074/jbc.M010434200 originally published online January 25, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010434200

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