Human DNA Polymerase \( \beta \) Recognizes Single-stranded DNA Using Two Different Binding Modes*

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Interactions between the human DNA polymerase \( \beta \) (pol \( \beta \)) and a single-stranded (ss) DNA have been studied using the quantitative fluorescence titration technique. Examination of the fluorescence increase of the poly(dA) etheno-derivative (poly(d(eA))) as a function of the binding density of pol \( \beta \)-nucleic acid complexes reveals the existence of two binding phases. In the first high affinity phase, pol \( \beta \) forms a complex with a ssDNA in which 16 nucleotides are occluded by the enzyme. In the second phase, transition to a complex where the polymerase occludes only 5 nucleotides occurs. Thus, human pol \( \beta \) binds a ssDNA in two binding modes, which differ in the number of occluded nucleotide residues. We designate the first complex as (pol \( \beta \))\(_{16}\) and the second as (pol \( \beta \))\(_{5}\) binding modes. The analyses of the enzyme binding to ssDNA have been performed using statistical thermodynamic models, which account for the existence of the two binding modes of the enzyme, cooperative interactions, and the overlap of potential binding sites. The importance of the discovery that human pol \( \beta \) binds a ssDNA, using different binding modes, for the possible mechanistic model of the functioning of human pol \( \beta \), is discussed.

Polymerase \( \beta \) (pol \( \beta \))\(^1\) is one of the four recognized DNA-directed polymerases of the eucaryotic nucleus: \( \alpha \), \( \beta \), \( \delta \), and \( \epsilon \) (1–3). The enzyme is lacking intrinsic accessory activities, such as 3’ or 5’ exonuclease, endonuclease, dNMP turnover, and pyrophosphorylation (1–5). This “simplified” activity reflects a very specialized function of the polymerase in mammalian cell repair machinery. Pol \( \beta \) conducts “gap fillings” synthesis on gapped DNA in a processive fashion (2, 4–6). The \( \textit{in vitro} \) gap filling reaction has been proposed as being consistent with the role of pol \( \beta \) in the gap filling synthesis involved in mismatch repair (4, 5, 7) and in the repair of nonfunctional adducts, UV damaged DNA, and abasic lesions in DNA (8–12).

Human pol \( \beta \) is a single polypeptide of \( \sim 39,000 \) kDa (13–16). Recently, crystallographic structures of rat and human pol \( \beta \) have been determined at 3.6- and 2.3-Å resolutions (15, 16). A characteristic feature of the pol \( \beta \) structure is the presence of a small 8-kDa domain, which is connected with the tip of the fingers through a tether of 14 amino acids (15, 16).

Solution studies showed that the 8-kDa domain has a high affinity for a ss nucleic acid, thus providing evidence that it is the template-binding domain, while the catalytic activity and dsDNA affinity reside in the large 31-kDa domain (17, 18).

The quantitative analysis of the interactions of human pol \( \beta \) with a ssDNA has not been directly addressed before. The only information about the interactions of the enzyme with a ss nucleic acid comes from studies of the interactions of an analogous rat enzyme with the fluorescent polynucleotide, poly(reA) (17, 18). These studies indicated that the enzyme forms a single type of complex with \( \sim 11 \) nucleotide residues occluded by the protein (18). The \( \textit{in vivo} \) role of pol \( \beta \) is related to its ability to fill short ssDNA gaps, \(< 6 \) nucleotides, during nucleotide excision repair (2, 3, 5, 19, 20). Fundamental questions of the pol \( \beta \) mechanism relate to the problem of how the enzyme, which is proposed to form a single type of complex with a ssDNA (18), efficiently recognizes small ssDNA gaps, much smaller than the indicated site size of the complex. Another important question is: does the enzyme have the ability to adjust to the decreasing accessibility of the ssDNA in the vanishing gap during DNA synthesis?

Elucidation of the pol \( \beta \)-ssDNA recognition processes constitutes a first step toward understanding the molecular mechanism of the enzyme. In this article, we report the quantitative analysis of human pol \( \beta \) interactions with the ssDNA using the thermodynamically rigorous fluorescence titration technique (21–24). We provide direct evidence that human pol \( \beta \) binds the ssDNA in two binding modes, (pol \( \beta \))\(_{16}\) and (pol \( \beta \))\(_{5}\), which differ in the number of occluded nucleotide residues in the complex. Thus, the enzyme can switch between high and low site size binding modes. This ability may be crucial for efficient recognition of the small ssDNA gaps on damaged DNA and for a processive DNA synthesis. Both binding modes differ in affinities and abilities to induce conformational changes in the ssDNA. These differences strongly suggest that, in the (pol \( \beta \))\(_{16}\) mode, the 31-kDa catalytic domain of the enzyme is involved in interactions with the ssDNA.

EXPERIMENTAL PROCEDURES

Reagents and Buffers—All chemicals were reagent grade. All solutions were made with distilled and deionized \( > 18 \) M\( \Omega \) (Milli-Q Plus) water. Buffer C is 10 mM sodium cacodylate adjusted to pH 7.0 with HCl, 1 mM MgCl\(_2\), and 10% glycerol. The temperatures and concentrations of NaCl in the buffer are indicated throughout the text.

Human Polymerase \( \beta \)—Human pol \( \beta \) was purified using a previously published procedure (14, 17). The concentration of the protein was determined using extinction coefficient \( \epsilon_{280} = 2.1 \times 10^{4} \) cm\(^{-1}\) M\(^{-1}\) obtained by the Edelhoch method (25, 26).

Nucleic Acids—All nucleic acids were purchased from Midland (Midland, TX). The etheno-derivatives of nucleic acids were obtained by modification with chloroacetaldehyde (27, 28). This modification goes to completion providing fluorescent derivative of a nucleic acid (27, 28). The concentrations of poly(dA(eA)) and poly(A(eA)) were spectrophotometrically determined using extinction coefficient 3700 M\(^{-1}\) cm\(^{-1}\) (nucleotide) at 257 nm (21–23, 28).

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‡ The abbreviations used are: pol \( \beta \), polymerase \( \beta \); ss, single-stranded; ds, double-stranded; MCT, macromolecular competition titration.
Fluorescence Measurements—All steady-state fluorescence titrations were performed as described previously by us (21–24). The binding was followed by monitoring the fluorescence of the etheno-derivatives, poly(deA) and deApeA15. Computer fits were performed using Mathematica (Wolfram, IL). The relative fluorescence increase of the nucleic acid, $\Delta F$, upon binding pol $\beta$ is defined as $\Delta F = (F - F_0)F_0$, where $F_0$ is the fluorescence of the nucleic acid at a given titration point $"i"$ and $F$ is the initial value of the fluorescence of the nucleic acid sample.

**Determination of Rigorous Thermodynamic Binding Isotherms and Stoichiometries of Human pol $\beta$-ssDNA Complexes**—In this work, we followed the binding of human pol $\beta$ to ssDNAs by monitoring the fluorescence increase, $\Delta F$, of their etheno-derivatives upon the complex formation. The method to obtain rigorous estimates of the average binding density, $\Sigma n_i$ (number of protein molecules bound per nucleotide) and the free protein concentration, $P_F$, has been previously described in detail by us (21–24). Briefly, the experimentally observed $\Delta F$ has a contribution from each of the different possible "i" complexes of human pol $\beta$ with a ssDNA. Thus, the observed fluorescence increase is functionally related to $\Sigma n_i$ by Equation 1.

$$\Delta F = \Sigma n_i \Delta F_{i\infty}$$ (Eq. 1)

$\Delta F_{i\infty}$ is the molecular parameter characterizing the maximum fluorescence increase of the nucleic acid with human pol $\beta$ bound in complex "i." The same value of $\Delta F$ obtained at two different total nucleic acid concentrations, $N_{Y1}$ and $N_{Y2}$, indicates the same physical state of the nucleic acid, i.e., the degree of binding, $\Sigma n_i$, and the free pol $\beta$ concentration, $P_F$, must be the same. The values of $\Sigma n_i$ and $P_F$ are then related to the total protein concentrations, $P_T$ and $P_{TF}$, and the total nucleic acid concentrations, $N_{T1}$ and $N_{T2}$, at the same value of $\Delta F$, by Equations 2a and 2b.

$$\Sigma n_i = (P_T - P_F)(N_{T2} - N_{T1})$$ (Eq. 2a)

$$P_F = P_{TF} - (\Sigma n_i)N_{T1}$$ (Eq. 2b)

$x = 1$ or 2 (21–24).

**Determination of the Thermodynamically Rigorous Isotherms of Human pol $\beta$ Binding to Unmodified ssDNA Homopolymers Using the MCT Method**—Determination of the interaction parameters for the human pol $\beta$-modified nucleic acid complexes has been performed using the macromolecular competition titration (MCT) method, recently developed by us (23), using the 16-mer, deApeA15, as a fluorescent reference nucleic acid. In this method, the fluorescent reference nucleic acid (deApeA15) at total concentration, $N_{PF}$, is titrated with the protein in the presence of a competing nonfluorescent nucleic acid (e.g., poly(dA)) at total concentration, $N_{TP}$, the total concentration of the protein, $P_T$, at which the same value of the relative fluorescence increase, $\Delta F$, of deApeA15, is observed in the absence of the unmodified ssDNA, $P_{TF}$, and in the presence of the unmodified ssDNA, $P_{TF}$, are defined (23) as shown in Equation 3 (a and b).

$$P_T = \Theta_{1b}N_{T1} + P_F$$ (Eq. 3a)

$$P_{TF} = \Theta_{1b}N_{T1} - (\Sigma n_i)N_{T1} + P_F$$ (Eq. 3b)

$\Theta_{1b}$, $\Sigma n_i$, and $P_F$ are the degree of binding of pol $\beta$ on deApeA15, the binding density of the protein on the nonfluorescent competing nucleic acid, and the free protein concentration, respectively. Solving the set of equations, 3a and 3b, for $\Sigma n_i$ and $P_F$ provides the thermodynamically rigorous binding density of human pol $\beta$ on an unmodified ssDNA and the free protein concentration, which are defined by Equations 3c and 3d (23).

$$\Sigma n_i = \frac{(P_T - P_{TF})}{(N_{T1})}$$ (Eq. 3c)

$$P_F = P_{TF} - (\Sigma n_i)N_{T1} - \Theta_{1b}N_{T1}$$ (Eq. 3d)

The theoretical formula for the degree of binding of human pol $\beta$ on deApeA15, $\theta_{1b}$, has been derived in this work and is defined by Equation 11 (see "Results").

**RESULTS**

**Statistical Thermodynamic Model for the Cooperative Binding of a Large Ligand in Two Different Binding Modes to a One-dimensional Infinite, Homogeneous Lattice**—The simplest statistical thermodynamic model that describes the binding of a large ligand, which occludes a number of $n$ nucleotides in the complex, to an infinite, homogeneous lattice is the McGhee-von Hippel model (29). This paradigm model allows one to extract an intrinsic binding constant, $K_1$, a parameter, $\omega$, which characterizes cooperative interactions between bound ligand molecules, and takes into account the overlap among potential binding sites. Previously, we derived a single, generalized equation for the McGhee-von Hippel model, which can be applied to both cooperative and noncooperative binding (30). The binding density, $\Sigma n_i$, is then described by Equation 4.

$$\Sigma n_i = K_1\left\{\frac{2\omega(1 - \Sigma n_i)}{(2\omega -1)(1 - \Sigma n_i) + \Sigma n_i + R}\right\}^{\frac{1}{1-\omega}} \left\{\frac{1 - (n + 1)\Sigma n_i + R}{2(1 - n \Sigma n_i)}\right\}^2 P_F$$ (Eq. 4)

where $R = [(1 - n + 1)\Sigma n_i + 4\omega\Sigma n_i(1 - n \Sigma n_i)]^{0.5}$.

Thus, in this model, a nucleic acid residue can only exist in two states, free and bound with the ligand. On the other hand, as we show below, pol $\beta$ binds the ssDNA in two binding modes, (pol $\beta$)$_{16}$ and (pol $\beta$)$_2$, differing by the number of occluded nucleotides (see below). Therefore, the formation of the complex between human pol $\beta$ and the ssDNA includes three states of the nucleic acid residues. A general secular equation for the three-state binding model has been previously derived by us (30). For pol $\beta$, which binds ssDNAs in two modes, this secular equation becomes

$$f(x) = P_F^2[K_1\Theta_{1b}K_2[(\omega_1\omega_2 - \omega_3\omega_2)x + (\omega_1 + \omega_2 - 2\omega_2 - \omega_1 \omega_2 + \omega_3)]$$

$$+ P_F[K_1[(\omega_2 - 1)x^n - \omega_1 \omega_2x^{n-1}] + K_2[(\omega_1 - 1)x^n - \omega_1 \omega_2x^{n-1}] + x^{n+1}] - x^{n+1} = 0, \quad (Eq. 5)$$

where $K_1$, $K_2$, and $K_3$ are the intrinsic binding constants for the (pol $\beta$)$_{16}$ and (pol $\beta$)$_2$, binding modes, $\omega_1$ and $\omega_2$ are the parameters characterizing cooperative interactions in the (pol $\beta$)$_{16}$ and (pol $\beta$)$_2$ modes, and $\omega_0$ characterizes the cooperative interactions between the enzyme molecules bound in the two different modes, (pol $\beta$)$_{16}$ and (pol $\beta$)$_2$. Additionally, in the case of human pol $\beta$, $n = 16$ and $m = 5$.

Secular Equation 5 defines the eigenvalues, $x$, of the ligand-macromolecule system. The grand partition function, $Z$, of the protein-ss nucleic acid system, where the nucleic acid is a long polymer of $N$ residues, can be written as $Z = x_N$, where $x_N$ is the largest root (largest eigenvalue) of secular Equation 5 (30–32). On the other hand, Equation 5 is a second degree polynomial, with respect to the free protein concentration, $P_F$. This equation has a physically realistic solution, evaluated at $x_1$.

$$P_F = [-p - (p^2 - 4q)^{0.5}/2q]$$ (Eq. 6)

where

$$p = K_1[(\omega_2 - 1)x^n - \omega_1 \omega_2x^{n-1}] + K_2[(\omega_1 - 1)x^n - \omega_1 \omega_2x^{n-1}]$$ (Eq. 7a)

and

$$q = K_1\Theta_{1b}K_2[(\omega_2 - 1)x^n + (\omega_1 + \omega_2 - 2\omega_2 - \omega_1 \omega_2 + \omega_3)]$$ (Eq. 7b)

and

$$r = x^{n+1} - x^n.$$ (Eq. 7c)

Thus, the average protein binding density, $\Sigma n_i$, is then defined by Equation 8.

$$\Sigma n_i = \left[\frac{\partial f(x)}{\partial P_F}\right] \left[\frac{\partial n(x)}{\partial x^{n+1}}\right]^{-1}$$ (Eq. 8)

For a given set of interaction parameters (intrinsic binding constants and cooperativity parameters), $P_F$ in Equation 6 is a sole function of $x_1$. Additionally, substituting Equation 6 into
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Fig. 1. Theoretical dependence of the observed fluorescence change, $\Delta F$, upon the total binding density, $\Sigma v_j$, for the binding of a large ligand in two cooperative binding modes, differing by the number of the occluded nucleotide residues to an infinite, homogeneous nucleic acid. Binding of the ligand in the first mode is characterized by the site size $n = 16$, cooperativity parameter $\alpha_1 = 3$, and different values of the intrinsic binding constant $K_{16}$. Binding of the ligand in the second mode is characterized by the site size $m = 5$, intrinsic binding constant $K_m = 10^3 M^{-1}$, and cooperativity parameter $\alpha_2 = 10$. The cooperativity parameter characterizing the interactions between the ligand molecules bound in different binding modes is $\alpha = 5$. Equation 8 renders $\Sigma v_j$ as a sole function of $x_1$. Thus, Equations 6 and 8 constitute a set of two parametric equations that completely define the binding isotherm with a single variable parameter, $x_1$. Both equations provide an easy way to perform computer simulations and/or fittings of the binding isotherms by treating $x_1$ as an independent variable. In the limit of an infinite lattice, $x_1$ is an effective partition function of a single lattice monomer. Therefore, $x_1$ can only assume real values from (1, $\infty$). Computer simulations can be performed by introducing $x_1$ into Equation 6 and calculating the corresponding $\{P_{j\lambda}\}$. Next, one can introduce $x_1$ and the $\{P_{j\lambda}\}$ into Equation 8, thus obtaining the required average value of the binding density, $\Sigma v_j$.

In our studies, we monitored the binding of human pol $\beta$ to a ssDNA in two different binding modes by monitoring the fluorescence increase of the nucleic acid, poly(deA). In order to illustrate the general feature of such a complex binding system, the theoretical dependence of the observed fluorescence change accompanying the binding of a protein to a ssDNA upon the total binding density, $\Sigma v_j$, of the protein on the nucleic acid is shown in Fig. 1. In these simulations the protein is assumed to bind in two weakly cooperative binding modes with site sizes of $n = 16$ and $m = 5$, respectively, and with different intrinsic affinities in both binding modes. We selected the maximum fluorescence change for the high site size binding mode, $\Delta F_{\text{max}(16)}$, as 2, and the corresponding change for the low site size mode, $\Delta F_{\text{max}(5)}$, as 2.5, respectively. The plots show characteristic nonlinear behavior. The isotherms are composed of two binding phases, particularly for the higher intrinsic affinities of the high site size binding mode. There is a strong inflection point between the two phases, which occurs at the binding density value corresponding to the site size of the high site size binding mode, i.e., $\Sigma v_{16} \approx 0.07$. This inflection point is still clearly visible even when the intrinsic binding constant of the high site size mode is, only by a factor of ~3, higher than the binding constant of the low site size mode (Fig. 1).

On the other hand, extrapolation of the high binding density region of the isotherms in Fig. 1 provides the stoichiometry of the low site size binding mode. Notice, in the considered case, the slope of the plot corresponding to this mode is not strictly linear, due to the differences in the relative fluorescence changes induced upon the complex formation with the nucleic acid in both binding modes. However, the obtained estimate of the site size is within $\pm 5\%$ of its true value. In the case of the experimental isotherms, such an error is completely absorbed by the inherent error of the data, which allows the site size of the low site size binding mode to be determined within $\pm 20\%$ of its true value (see below).

Stoichiometry of Human pol $\beta$-ssDNA Complexes—Previous studies showed that binding of analogous rat pol $\beta$ to the fluorescent polyribonucleotide, poly(rA), is accompanied by a large nucleic acid fluorescence increase (18). The fluorescence increase of the etheno-derivative of a nucleic acid in the complex with a protein is dependent upon the salt concentration in solution, particularly Mg$^{2+}$ cations (21–24). We have found that binding of human pol $\beta$ to the ssDNA fluorescence derivative, poly(deA), causes a strong nucleic acid fluorescence increase even in the presence of 1 mM MgCl$_2$. Fluorescence titrations of poly(deA) with human pol $\beta$ at two different nucleic acid concentrations, in buffer C (pH 7.0, 10°C) containing 50 mM NaCl, are shown in Fig. 2. The relative increase of the nucleic acid fluorescence reaches the value of 2.3 $\pm$ 0.2. At higher nucleic acid concentrations, a given fluorescence increase is reached at higher enzyme concentrations, due to the extra nucleic acid in the solution. The selected nucleic acid concentrations provide separation of the isotherms up to the relative fluorescence increase of $\approx 2$, i.e., up to $\approx 85\%$ of the binding curve.

To obtain thermodynamically rigorous binding isotherms, independent of any assumption about the relationship between the observed signal and the binding density, $\Sigma v_j$, the titration curves in Fig. 2 have been analyzed, using the approach outlined in Experimental Procedures. Fig. 3a shows the dependence of the observed relative fluorescence increase as a function of the average binding density, $\Sigma v_j$, of the enzyme. The plot is nonlinear and clearly shows two binding phases. In the first phase, at low protein concentrations, the binding density reaches the value of 0.06 $\pm$ 0.005, which corresponds to the site size of the enzyme-ssDNA complex of 16 $\pm$ 2 nucleotide resi-
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Fig. 3. a, the dependence of the relative fluorescence change upon the binding density, $\Sigma V_i$, of human pol $\beta$ on poly(deA) in buffer C (pH 7.0, 10 °C) containing 50 mM NaCl (■). The thermodynamically rigorous binding density, $\Sigma V_i$, has been determined using the quantitative method described under “Experimental Procedures” (21–24). The solid lines are tangent to the slopes corresponding to the low and high binding density phases of the isotherm. The intersection of the lines occurs at $V_i = 0.06 \pm 0.005$ and indicates the stoichiometry of the high site size (pol $\beta_{16}$) binding mode. The dashed line is an extrapolation of the high binding density phase to the maximum value of the relative fluorescence change, $\Delta F_{\text{max}} = 2.3$, which provides the stoichiometry, $V_i = 0.22 \pm 0.03$ of the low site size (pol $\beta_{5}$) binding mode. $b$, the dependence of the binding density, $\Sigma V_i$, of the pol $\beta$-poly(deA) complex upon the logarithm of the total enzyme concentration in buffer C (pH 7.0, 10 °C) containing 50 mM NaCl (■). The concentration of poly(dA)dT is $6.67 \times 10^{-6}$ M (nucleotide). The solid line is the computer fit of the initial part of the isotherm, obtained by applying the theory and methodology described in the text, using the model of two cooperative binding modes, (pol $\beta_{16}$) and (pol $\beta_{5}$) (Equations 6–8), with $K_{\text{16}} = 9 \times 10^9$ M$^{-1}$, $K_{\text{5}} = 3 \times 10^9$ M$^{-1}$, $\omega_1 = 1$, $\omega_2 = 3$, and $\omega_5 = 2$. The dashed line is the computer fit of the initial part of the isotherm, corresponding to the (pol $\beta_{16}$) mode, using the generalized McGhee-von Hippel Equation 4, with $K_{\text{16}} = 9 \times 10^9$ M$^{-1}$ and $\omega_1 = 1$.

In the second phase, at higher protein concentrations, short extrapolation of the degree of binding to the maximum value of the relative fluorescence change provides a stoichiometry of $3.2 \pm 0.3$ of the human pol $\beta$-16-mer system. Thus, three pol $\beta$ molecules bind per 16-mer at saturation, which indicates the formation of the (pol $\beta_{16}$) mode. These results are in excellent agreement with the results obtained with the polymer ssDNA.

**Intrinsic Affinities and Cooperativities in the Interactions of Human pol $\beta$ with a ssDNA in the (pol $\beta_{16}$) and (pol $\beta_{5}$) Binding Modes**—In order to obtain estimates of the intrinsic affinity and cooperativity of human pol $\beta$ binding to the ssDNA in different binding modes, the titration curves of poly(deA) with human pol $\beta$ have been analyzed, using the statistical thermodynamic model described above. With the independent estimates of the size of the two binding modes, there are five remaining interaction parameters, $K_{\text{16}}$, $K_{\text{5}}$, $\omega_1$, $\omega_2$, and $\omega_5$, and two fluorescence changes accompanying the binding in two modes, $\Delta F_{\text{max(16)}}$ and $\Delta F_{\text{max(5)}}$, to be determined. This is still a formidable number of parameters, which precludes any attempt to obtain these quantities in a single fitting procedure.

We apply the following strategy to determine all interaction and spectroscopic parameters for this very complex binding system. Inspection of the thermodynamic isotherm in Fig. 3a shows that the (pol $\beta_{16}$) binding mode is significantly separated from the (pol $\beta_{5}$) mode, with respect to the free protein concentration, due to the higher macroscopic affinity of the (pol $\beta_{16}$) mode. Such separation of the binding modes allows us to independently obtain estimates of $K_{\text{16}}$ and $\omega_1$, due to the fact that, initially, binding in the (pol $\beta_{16}$) mode completely dominates the association process. This is achieved by analyzing the initial part of the thermodynamic isotherm, such as in Fig. 3b, where there is exclusive binding in the (pol $\beta_{16}$) mode, using the generalized McGhee-von Hippel isotherm, as defined by Equation 4. The dashed line in Fig. 3b is the computer fit of the initial part of the binding isotherm using the McGhee-von Hippel model with $n = 16$ and $K_{\text{16}}$ and $\omega_1$ as fitting parameters.
of the tangent lines indicates the stoichiometry of 1.1 lines rigorous method described under "Experimental Procedures." The degree of binding have been determined using the thermodynamically divided in the text, are standard deviations obtained using five or six mm NaCl, at two different concentrations of the nucleic acid (oligomer): ■ 2.13 × 10^{-6} M; □ 2.13 × 10^{-7} M. The solid lines are computer fits of the experimental fluorescence binding isotherms according to the model of the two binding modes (Equations 9–11), using a single set of parameters: \( K_5 = 7 × 10^{-6} \) M\(^{-1}\), \( K_6 = 1 × 10^{-5} \) M\(^{-1}\), \( \omega_3 = 15 \), \( \Delta F_{\text{max}(16)} = 0.56 \), and \( \Delta F_{\text{max}(5)} = 1.23 \). Errors in determining the parameters, provided in the text, are standard deviations obtained using five or six independent titration experiments. 

b, the dependence of the relative fluorescence increase upon the degree of binding of the human pol β-16-mer complex. The thermodynamically rigorous values of the degree of binding have been determined using the thermodynamically rigorous method described under “Experimental Procedures.” The solid lines are the limiting slopes of the two binding phases. 

The intersection of the tangent lines indicates the stoichiometry of 1.1 ± 0.1 for the high site size (pol β)\(_{16}\) complex. The dashed line is an extrapolation of the degree of binding to the maximum value of the observed fluorescence increase \( \Delta F_{\text{max}} = 1.2 \), which provides the maximum stoichiometry of 3.2 ± 0.3 of the human pol β-16-mer complex.

For the isotherm in Fig. 3b, we obtained \( K_{16} = (9 ± 4) × 10^{-7} \) M\(^{-1}\) and \( \omega_3 = 1.0 ± 0.5 \). The fitting analysis has been performed using several different isotherms, obtained at several different nucleic acid concentrations. The results indicate very little, if any, cooperative interactions in the (pol β)\(_{16}\) binding mode (data not shown). With the estimates of \( K_{16} \) and \( \omega_3 \), the isotherm in Fig. 3b is further subjected to a nonlinear fit, to obtain estimates of the only three remaining interaction parameters, \( K_5 \), \( \omega_2 \), and \( \omega_0 \). The fit provides \( K_5 = (3 ± 1.5) × 10^{-6} \) M\(^{-1}\), \( \omega_2 = 3 ± 1 \), and \( \omega_0 = 2 ± 1 \). The results indicate that the intrinsic binding constant for the (pol β)\(_7\) mode is = −2 orders of magnitude lower as compared with the intrinsic binding constant of the (pol β)\(_{16}\) mode. Additionally, the data indicate the lack of any significant cooperative interactions within the (pol β)\(_3\) binding mode, as well as between the (pol β)\(_{16}\) and (pol β)\(_7\) modes.

The spectroscopic parameters are obtained by fitting the fluorescence titration curves with \( \Delta F_{\text{max}(16)} \) and \( \Delta F_{\text{max}(5)} \) as the only remaining fitting parameters. The solid lines in Fig. 2 are the computer best fits of the fluorescence titration curve, using the interaction parameters obtained from the analysis of the thermodynamic isotherm described above, which provides \( \Delta F_{\text{max}(16)} = 1.7 ± 0.2 \) and \( \Delta F_{\text{max}(5)} = 2.3 ± 0.2 \). Thus, the fluorescence change accompanying the binding in the (pol β)\(_{16}\) mode is significantly lower than the corresponding fluorescence change accompanying the formation of the (pol β)\(_7\) binding mode.

Statistical Thermodynamic Model of Human pol β Binding to the 16-mer, deA(peA)\(_{15}\)—Binding of human pol β to deA(peA)\(_{15}\) proceeds by the formation of a 1:1 complex, corresponding to the (pol β)\(_{16}\) binding mode, which is subsequently replaced by a complex in which three pol β molecules are bound in the (pol β)\(_5\) mode (Fig. 4b). Notice, there are no interactions between the protein molecules bound in the (pol β)\(_{16}\) and (pol β)\(_3\) binding modes in the complex with the 16-mer. Therefore, binding of pol β to deA(peA)\(_{15}\) in the (pol β)\(_{16}\) mode is described by a single-site binding isotherm with the degree of binding \( v = K_{16} P_b/(1 + K_{16} P_b) \). To derive a part of the partition function corresponding to the binding of pol β in the (pol β)\(_5\) mode and to formulate a complete partition function, we used the Epstein theory for binding a large ligand to a finite lattice (23, 32, 33). The complete partition function of the human pol β-deA(peA)\(_{15}\) system, \( Z_{16} \) is given in Equation 9.

\[
Z_{16} = 1 + K_{16} P_b + \sum_{k=1}^{g} \sum_{j=0}^{k-1} S_{ij}(k,j)(K_{16} P_b)^{k} \omega_{j} \quad (\text{Eq. 9})
\]

\( g \) is the maximum number of human pol β molecules that can bind to a 16-mer at saturation (in our case, \( g = 3 \)), \( k \) is the number of protein molecules bound at a given \( P_b \), and \( j \) is the number of cooperative contacts between the \( k \) bound human pol β molecules in a particular configuration on the lattice. The factor \( S_{ij}(k,j) \) is the number of distinct ways that \( k \) ligands bind to a lattice, with \( j \) cooperative contacts, and is defined by Equation 10 (33).

\[
S_{ij}(k,j) = \frac{[(M - m k + 1)(k - 1)!]}{[(M - m k + j + 1)(k - j)(k - j - 1)!]} \quad (\text{Eq. 10})
\]

The total degree of binding, \( \theta_{16} \), is then defined as shown in Equation 11.

\[
\theta_{16} = [K_{16} P_b + \sum_{k=1}^{g} \sum_{j=0}^{k-1} k S_{ij}(k,j)(K_{16} P_b)^{k} \omega_{j}]/Z_{16} \quad (\text{Eq. 11})
\]

The interactions of human pol β with deA(peA)\(_{15}\) are characterized by only three interaction parameters, \( K_{16} \), \( K_5 \), and \( \omega_3 \). In the first step of estimating these parameters, the thermodynamic isotherm (\( \theta_{16} \), as a function [pol β]_{total} is subjected to nonlinear fitting providing \( K_{16} = (7 ± 3) × 10^{-9} \) M\(^{-1}\), \( K_5 = (1 ± 0.5) × 10^{-6} \) M\(^{-1}\), and \( \omega_3 = 15 ± 5 \). The data indicate that both intrinsic binding constants are lower than the value obtained in the studies with the polymer ssDNA in the same solution conditions (see above). This difference may reflect the fact that, in the polymer ssDNA, the binding site is embedded in the long polymer, while in the case of the oligomer, the same fragment of nucleic acid may have a slightly different conformational state, particularly at both ends, even in the complex with the protein, leading to less efficient interactions, particularly in the (pol β)\(_{16}\) mode. On the other hand, \( K_{16} \) is still significantly higher than \( K_5 \), as observed in the case of the poly(deA)-pol β

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**Fig. 4.** a, fluorescence titrations (λ\(_{em} = 325\) nm, λ\(_{ex} = 410\) nm) of deA(peA)\(_{15}\) with human pol β in buffer C (pH 7.0, 10 °C) containing 50 mm NaCl, at two different concentrations of the nucleic acid (oligomer): ■ 2.13 × 10^{-6} M; □ 2.13 × 10^{-7} M. The solid lines are computer fits of the experimental fluorescence binding isotherms according to the model of the two binding modes (Equations 9–11), using a single set of parameters: \( K_5 = 7 × 10^{-6} \) M\(^{-1}\), \( K_6 = 1 × 10^{-5} \) M\(^{-1}\), \( \omega_3 = 15 \), \( \Delta F_{\text{max}(16)} = 0.56 \), and \( \Delta F_{\text{max}(5)} = 1.23 \). Errors in determining the parameters, provided in the text, are standard deviations obtained using five or six independent titration experiments. b, the dependence of the relative fluorescence increase upon the degree of binding of the human pol β-16-mer complex.
buffer C (pH 7.0, 10 °C) containing different NaCl concentrations:

...indicating a decreasing macroscopic affinity of the human pol

...which provide...

...than the corresponding parameters obtained for polymer

...obtained with poly(dA) (2.09

...model of the two cooperative binding modes described by Equations 6–9 (Table I). The concentration of poly(dA) is 6.67 × 10⁻⁵ M (nucleotide). b, the dependence of the intrinsic binding constants, \( K_{b1} \) and \( K_{b2} \), for the binding of human pol β to poly(dA) upon NaCl concentration (log-log plots) in buffer C (pH 7.0, 10 °C) containing 1 mM MgCl₂.

 complexes, indicating that the differences in the energetics of the interactions of the enzyme with the ssDNA in the (pol β)₁₆ and (pol β)₃ modes are preserved in the complexes with the oligomer (see “Discussion”). Additionally, although \( \omega_2 \) is higher than the corresponding parameters obtained for polymer ssDNA, its value (15 ± 6) still indicates the lack of significant cooperative interactions within the (pol β)₃ mode. The spectroscopic parameters for the complexes with deA(peA)₁₅ are obtained by fitting the fluorescence titration curves with \( \Delta F_{\max(16)} \) and \( \Delta F_{\max(5)} \) (as the remaining fitting parameters), which provides \( \Delta F_{\max(16)} = 0.6 ± 0.1 \) and \( \Delta F_{\max(5)} = 1.2 ± 0.1 \) (Fig. 4a). Notice, although these values are lower than the corresponding values obtained with poly(dA), the fluorescence change accompanying the binding in the (pol β)₁₆ mode in the 16-mer complex is lower than the corresponding fluorescence change accompanying the formation of the (pol β)₃ mode with the oligomer, analogously to the results obtained with the polymer ssDNA.

Salt Effect on Intrinsic Affinities of (pol β)₁₆ and (pol β)₃ Binding Modes in the Complexes with the 16-mer, deA(peA)₁₅—Fluorescence titrations of deA(peA)₁₅ with human pol β, in buffer C (pH 7.0, 10 °C) containing different NaCl concentrations, are shown in Fig. 6a. The isotherms shift toward higher total enzyme concentrations at high salt concentrations, indicating a decreasing macroscopic affinity of the human pol β-nucleic acid complex. The dependence of the logarithm of the intrinsic binding constant upon the logarithm of [NaCl] (log-log plot) is shown in Fig. 5b (34). Within experimental accuracy, both plots are linear in the studied salt concentration ranges. However, there is a significant difference between the slopes obtained for the (pol β)₁₆ and (pol β)₃ modes. In the case of the (pol β)₁₆ mode, the slope, \( \Delta \log K_{a6}/\log [\text{NaCl}] \), is \(-6 ± 1\), which is 2-fold larger than \( \Delta \log K_{a3}/\log [\text{NaCl}] = -2.9 ± 0.6 \) obtained for the (pol β)₃ binding mode. Thus, although both intrinsic binding processes are accompanied by a net release of ions, the number of ions released in the formation of the (pol β)₁₆ mode is twice as large as compared with the (pol β)₃ mode, which most probably reflects the increased number of ionic contacts between the protein and the nucleic acid in the high site size binding mode. Notice, the larger number of ions released in the formation of the (pol β)₁₆ binding mode indicates that this mode will be much less favorable at a higher salt concentration (Fig. 5b). The contrary is true for the (pol β)₃ mode, which at salt concentrations >0.15 M begins to dominate the binding process. The small values of cooperativity parameters and the inherent large error in their determinations hinder a quantitative determination of the salt effect (Table I). Nevertheless, the obtained values of \( \omega_1 \), \( \omega_2 \), and \( \omega_3 \) indicate little dependence on the salt concentrations (Table I).

Salt Effect on Intrinsic Affinities of Human (pol β)₁₆ and (pol β)₃—Fluorescence titrations of dA(peA)₁₅ with human pol β, in buffer C (pH 7.0, 10 °C) containing different NaCl concentrations, are shown in Fig. 6a. The isotherms shift toward higher total enzyme concentrations at high salt concentrations, indicating a decreasing macroscopic affinity of the human pol β-nucleic acid complex. The dependence of the logarithm of the intrinsic binding constant upon the logarithm of [NaCl] (log-log plot) is shown in Fig. 6b. There is a clear difference between the number of ions released in both binding modes in the complexes with the polymer and oligomer ssDNAs. In the case of the (pol β)₁₆ mode, formed with the ssDNA 16-mer, \( \Delta \log K_{a6}/\log [\text{NaCl}] \) is only \(-3.9 ± 0.7\), as compared with \(-6\) in the case of poly(dA), while in the case of the (pol β)₃ binding mode, formed with the oligomer, the slope, \( \Delta \log K_{a3}/\log [\text{NaCl}] = -2.1 ± 0.5\), which is lower than \(-2.9\) obtained with the polymer ssDNA. The decrease of the number of ions released in the formation of both (pol β)₁₆ and (pol β)₃ binding modes, in the complex with the oligomer ssDNA, may result from the different possible conformational states of nucleotide residues at the ends of the short 16-mer leading to a decrease in the number of ionic contacts between the protein and the nucleic acid, as compared with the complexes with the polymer ssDNA. Additionally, due to its oligomeric nature, the thermodynamic degree of cation binding to the 16-mer should be lower, particularly at the ends of an oligomer, when compared with the polymer ssDNA. This property will lead to a lower number of ions released (35).

Base Specificity in Human pol β-ssDNA Interactions—Using the MCT method outlined under “Experimental Procedures” (33), we can quantitatively characterize the interactions between human pol β with unmodified polynucleotides, poly(dA), poly(dT), and poly(dC). Fluorescence titration of the deA(peA)₁₅ (2.13 × 10⁻⁶ M (oligomer)) with human pol β in the presence of poly(dT) (2.09 × 10⁻⁴ M (nucleotide)) in buffer C (pH 7.0, 10 °C) containing 150 mM NaCl, is shown in Fig. 7a. For comparison, the titration curve of only deA(peA)₁₅ with the polymerase, at the same concentration of the oligomer as in the titration...
performed in the presence of poly(dT), is also included. In the presence of the competing nonfluorescent nucleic acid, the binding isotherm is shifted toward higher protein concentrations due to the simultaneous binding of the protein to the competing, unmodified polynucleotide. On the other hand, at the same value of the fluorescence increase of dA(polyA)$_{15}$, in buffer C (pH 7.0, 10 °C) containing 150 mM NaCl and 2.13 × 10$^{-6}$ M dA(polyA)$_{15}$, the dependence of the intrinsic binding density, $\Sigma v_{1}$, of the human pol $\beta$-poly(dT) complex, upon the logarithm of the total enzyme concentration in buffer C (pH 7.0, 10 °C) containing 150 mM NaCl and 2.13 × 10$^{-6}$ M dA(polyA)$_{15}$ is described by $K_{16} = 8 \times 10^{4} \text{ M}^{-1}$, $K_{0} = 1 \times 10^{4} \text{ M}^{-1}$, $\omega_{1} = 1$, $\omega_{2} = 3$, and $\omega_{3} = 1$ (Table III). The concentration values of dA(polyA)$_{15}$ and poly(dT) are 2.13 × 10$^{-6}$ M (oligomer) and 2.09 × 10$^{-4}$ M (nucleotide), respectively. In the presence of the competing polynucleotide, the physical state of the fluorescent nucleic acid must be the same, i.e. the values of $\theta_{i}$ and the free protein concentration [human pol $\beta$]$_{P}$ must be the same. The binding density of the protein, on the competing, unmodified ssDNA, $(\Sigma v_{1})/\Sigma$, is also a sole, unique function of the [human pol $\beta$]$_{P}$. Therefore, at a

![Image](50x174 to 294x590)

**Table I**

| Parameter | 50 | 75 | 100 | 150 |
|-----------|----|----|-----|-----|
| $K_{16}$ (M$^{-1}$) | $(9 \pm 4) \times 10^{7}$ | $(1 \pm 0.5) \times 10^{7}$ | $(1 \pm 0.5) \times 10^{7}$ | $(1 \pm 0.5) \times 10^{7}$ |
| $\omega_{1}$ | 1 | 0.5 | 1 | 0.5 |
| $K_{0}$ (M$^{-1}$) | $(3 \pm 1.5) \times 10^{9}$ | $(1.7 \pm 0.8) \times 10^{5}$ | $(1.5 \pm 0.6) \times 10^{5}$ | $(5.5 \pm 2.5) \times 10^{4}$ |
| $\omega_{2}$ | 3 | 1 | 2 | 1 |
| $\Delta F_{max(16)}$ | $1.7 \pm 0.2$ | $1.1 \pm 0.2$ | $1.1 \pm 0.2$ | $0.8 \pm 0.2$ |
| $\Delta F_{max(5)}$ | $2.3 \pm 0.2$ | $1.7 \pm 0.2$ | $1.6 \pm 0.2$ | $1.4 \pm 0.2$ |

![Image](312x221 to 549x582)

**FIG. 6.** (a) Fluorescence titrations of dA(polyA)$_{15}$ with human pol $\beta$ in buffer C (pH 7.0, 10 °C) containing different NaCl concentrations: ■, 50 mM; □, 75 mM; ○, 100 mM; △, 150 mM. The solid lines are computer fits of the binding isotherms using the combined single-site and Epstein’s model of a large ligand binding to a short nucleic acid lattice as described by Equations 9–11 (Table II). (b), the dependence of the intrinsic binding constants, $K_{16}$ (■) and $K_{0}$ (○), for the binding of human pol $\beta$ to dA(polyA)$_{15}$ upon NaCl concentration (log-log plots) in buffer C (pH 7.0, 10 °C) containing 1 mM MgCl$_2$.

**FIG. 7.** (a) Fluorescence titrations of dA(polyA)$_{15}$ with human pol $\beta$ in buffer C (pH 7.0, 10 °C) containing 150 mM NaCl, in the presence of poly(dT) (○). For comparison, the fluorescence titration of only dA(polyA)$_{15}$, in the same solution conditions, is included (■). The solid line is the computer fit of the experimental fluorescence binding isotherm according to the model of the two binding modes using: $K_{16} = 1 \times 10^{5} \text{ M}^{-1}$, $K_{0} = 7 \times 10^{3} \text{ M}^{-1}$, $\omega_{1} = 1$, $\omega_{2} = 3$, and $\omega_{3} = 1$ (Table III). The concentration values of dA(polyA)$_{15}$ and poly(dT) are 2.13 × 10$^{-6}$ M (oligomer) and 2.09 × 10$^{-4}$ M (nucleotide), respectively. In the presence of the competing polynucleotide, the physical state of the fluorescent nucleic acid must be the same, i.e. the values of $\theta_{i}$ and the free protein concentration [human pol $\beta$]$_{P}$ must be the same. The binding density of the protein, on the competing, unmodified ssDNA, $(\Sigma v_{1})/\Sigma$, is also a sole, unique function of the [human pol $\beta$]$_{P}$. Therefore, at a

**DNA Repair Polymerase-ssDNA Interactions**

Thermodynamic and spectroscopic parameters for human pol $\beta$ binding to poly(dA) in two binding modes, (pol $\beta_{16}$ and (pol $\beta_{1}$) in buffer C (pH 7.0, 10 °C) containing different NaCl concentrations

The errors are standard deviations determined using four to five independent titration curves.
given value of fluorescence increase, the value of \( \Sigma \nu_i \) must be the same, independent of the concentration of the unmodified nucleic acid. The binding density, \( \Sigma \nu_i \), can then be obtained using Equation 3c (see "Experimental Procedures").

The dependence of the determined binding density, \( \Sigma \nu_i \), of the pol \( \beta \)-poly(dT) complex, as a function of the total protein concentration, is shown in Fig. 7b. In the analysis of the thermodynamic binding isotherm of the pol \( \beta \)-unmodified ssDNA polymers, we use a similar strategy to extract all interaction parameters as applied in studying the binding to poly(dA) (see above). Because the binding in the (pol \( \beta \))\textsubscript{16} mode dominates the association process at the low protein concentrations, due to the higher macroscopic affinity than the (pol \( \beta \))\textsubscript{5} mode, we can independently obtain estimates of \( K_{\text{16}} \) and \( \omega_1 \). This is achieved by analyzing the initial part of the thermodynamic isotherm, such as in Fig. 7b, where there is exclusive binding in the (pol \( \beta \))\textsubscript{16} mode, using the generalized McGhee-von Hippel isotherm, as defined by Equation 4. The dashed line in Fig. 7b is the computer fit of the initial part of the binding isotherm using the McGhee-von Hippel model with \( n = 16 \) and \( K_{\text{16}} \) and \( \omega_1 \) as fitting parameters (Equation 4). For the binding to poly(dT), we obtained \( K_{\text{16}} = (1 \pm 0.5) \times 10^5 \text{ M}^{-1} \) and \( \omega_1 = 1.0 \pm 0.5 \) (buffer C, pH 7.0, 10 °C, 150 mM NaCl). This fitting analysis has been performed using several different isotherms, obtained at several different poly(dT) and deA(peA)\textsubscript{16} concentrations to obtain the most accurate estimates of the binding parameters (data not shown). With the estimates of \( K_{\text{16}} \) and \( \omega_1 \), the isotherm in Fig. 7b is further subjected to a nonlinear least-square fit to obtain estimates of the only three remaining interaction parameters, \( K_{\text{5}} \), \( \omega_2 \), and \( \omega_3 \). The fit provides \( K_{\text{5}} = (2 \pm 1) \times 10^4 \text{ M}^{-1}, \omega_2 = 4 \pm 2, \) and \( \omega_3 = 1 \pm 0.5 \) (Table III).

Fluorescence titrations of the deA(peA)\textsubscript{16} with human pol \( \beta \) in the presence of poly(dA), poly(dT), and poly(dC) in buffer C (pH 7.0, 10 °C) containing 150 mM NaCl, are shown in Fig. 8. The analysis of the binding isotherms obtained with different ssDNA polymers has been performed in the same way as described above for the binding of human pol \( \beta \) to poly(dT). The obtained parameters are included in Table III. The results indicate that in the case of poly(dA), poly(dT), and poly(dC) the enzyme has similar intrinsic binding constants in both (pol \( \beta \))\textsubscript{16} and (pol \( \beta \))\textsubscript{5} modes. However, the strikingly low intrinsic affinity in the (pol \( \beta \))\textsubscript{16} binding mode occurs in the case of poly(dA), where \( K_{\text{16}} \) is ~30-fold lower than the corresponding intrinsic binding constants for the other ssDNAs. In all studied systems, the binding of human pol \( \beta \) to ssDNA is characterized by very low cooperative interactions in both the (pol \( \beta \))\textsubscript{16} and (pol \( \beta \))\textsubscript{5} binding modes, as well as between different binding modes (Table III).

### DISCUSSION

**Human pol \( \beta \) Binds ssDNA in Two Binding Modes, (pol \( \beta \))\textsubscript{16} and (pol \( \beta \))\textsubscript{5}**

Differing in the Number of Occluded Nucleotide Residues in the Protein-DNA Complexes—Quantitative fluorescence titration studies described in this work provide direct evidence that human pol \( \beta \) binds the ssDNA using two binding modes, (pol \( \beta \))\textsubscript{16} and (pol \( \beta \))\textsubscript{5}, which differ in the number of occluded nucleotide residues in the protein-ssDNA complex. The discovery of the existence of the two binding modes of the intact enzyme, and the transition between them induced by the increase of the binding density of the protein on the nucleic acid lattice, was possible due to the application of the thermodynamically rigorous fluorescence titration technique, which allowed us to determine the binding density, \( \Sigma \nu_i \), of the protein-ssDNA complex, over a large protein concentration range, without any assumption about the relationship between the observed signal used to monitor the binding and the stoichiometry of the studied complexes (21–24). The characteristic dependence of the observed fluorescence change upon the \( \Sigma \nu_i \) reveals the existence of two binding phases. The first phase, at low protein concentrations, corresponds to the formation of the (pol \( \beta \))\textsubscript{16} mode, and the second phase, where the enzyme binds in the (pol \( \beta \))\textsubscript{5} mode, is observed at higher protein concentrations (Fig. 3). The formation of both (pol \( \beta \))\textsubscript{16} and (pol \( \beta \))\textsubscript{5} binding modes was further confirmed in experiments with the short ssDNA oligomer, deA(peA)\textsubscript{16} (Fig. 4).

We derived a closed form parametric formula for a general, statistical thermodynamic model, which describes the cooperative binding of a large ligand in two binding modes to an infinite, homogeneous lattice (31, 32). A corresponding model has been obtained for the enzyme binding to the 16-mer, using the exact combinatorial approach (33). These analytical equations for the large ligand-lattice system allow for direct computer simulations and fittings of complex binding isotherms, using the general model of two cooperative binding modes without the necessity for cumbersome numerical calculations. Additionally, using the derived parametric equations, the examination of the behavior of such binding systems, by computer simulations, is reduced to the same level as analyzing the binding of a ligand in a single binding mode. Application of these analytical models to the analysis of the binding of human pol \( \beta \) to polymer and oligomer ssDNAs allows us to extract all interaction and spectroscopic parameters, which include five interaction parameters \( (K_{\text{16}}, K_5, \omega_1, \omega_2, \omega_3) \) and two spectroscopic parameters \( (\Delta F_{\text{max}}(16)\text{ and } \Delta F_{\text{max}}(5))\).
tions with the ssDNA.

Additional evidence that the 31-kDa domain is involved in interactions with the ssDNA in the (pol β)$_{16}$ mode come from the fact that the fluorescence change accompanying the formation of the (pol β)$_{16}$ mode is lower than the fluorescence change resulting from the formation of the (pol β)$_{5}$ mode (Tables I and II). This is true for both complexes with polymer and oligomer ssDNAs. Independent binding studies of the isolated 8-kDa domain to poly(dA) show that, in given solution conditions, the fluorescence increase accompanying the binding is similar to the fluorescence increase observed for the (pol β)$_{5}$ mode. The fluorescence of εA is dramatically quenched, ~8–12-fold, in etheno-oligomers and poly(dA), as compared with the free εAMP (21–25, 36). It has been proposed that this strong quenching predominantly results from the intramolecular collision between εA bases (36). The different extent of the fluorescence increase in the protein-nucleic acid complexes most probably reflects the different extent of limiting these quenching processes, with the strong interactions being more efficient in restoring the εA fluorescence (21–24). Thus, if the formation of the (pol β)$_{16}$ mode includes both strong and weak interacting 8-kDa and 31-kDa domains, the observed fluorescence increase will be averaged over the strong and weak interactions with both domains, i.e., it will be lower as compared with the fluorescence change accompanying the formation of (pol β)$_{5}$ where, most probably, only the strongly interacting 8-kDa domain is engaged in the complex with the ssDNA (see below).

**Model for the Binding Mode Transition in Human pol β-ssDNA Complexes**—A characteristic feature of human pol β binding modes is that the transition between the modes is induced by the increased protein concentration, i.e. the increase of the protein binding density on the DNA. This means that when the availability of the ssDNA is decreasing, the protein, initially bound to 16 nucleotide residues in the (pol β)$_{16}$ mode, is forced to bind the nucleic acid with a lower 5-nucleotide site size. In this context, the simplified model of the binding mode transition in human pol β complexes with ssDNA, which takes into account the presence of two different binding modes and the domain structure of the enzyme, is shown in Fig. 9. At low protein concentrations, when the ssDNA is in large excess, the enzyme binds the nucleic acid with both 8- and 31-kDa domains engaged in the complex with the ssDNA. As we discussed above, the obtained data indicate that the 31-kDa domain is involved in the weak interactions with the ssDNA, despite the low intrinsic affinity, due to the close proximity of the nucleic acid in the complex leading to additional stabilization of the (pol β)$_{16}$ mode. As the protein binding density increases, the negative entropy factor of finding a long enough stretch of ssDNA able to accept the (pol β)$_{16}$ mode becomes very high (29). At high binding densities, the weak interactions of the 31-kDa domain with the ssDNA become overwhelmed by the negative lattice entropy and the protein binds the nucleic acid in the (pol β)$_{5}$ mode in which only an 8-kDa template-binding domain is bound to the ssDNA. This transition would happen despite the fact that the (pol β)$_{16}$ mode has a higher intrinsic affinity than the (pol β)$_{5}$ binding mode (29). Thus, in this model of the binding mode transition of human pol β-ssDNA complexes, the 8-kDa domain interacts with the ssDNA in both (pol β)$_{16}$ and (pol β)$_{5}$ binding modes (Fig. 9). The observed lower intrinsic binding constant of the (pol β)$_{5}$ mode would result from the lack of additional interactions between the ssDNA and the 31-kDa domain.

**Possible Role of the High and Low Site Size Binding Modes in Human pol β Interactions with Gapped DNA Substrates**—The ability of the enzyme to switch from a high site size mode to a low site size mode may play a fundamental role in the mechanism of human pol β functioning. As we pointed out, the large site size binding mode, (pol β)$_{16}$, predominates at low binding densities, i.e. when the ssDNA is in large excess over the protein, while the protein switches to a low site size mode as the nucleic acid becomes less and less available at a higher degree of saturation with the protein. Thus, the (pol β)$_{16}$ binding mode can be operational in DNA synthesis on large ssDNA gaps. The involvement of the 31-kDa catalytic domain in interactions with a ssDNA also suggests that the DNA synthesis on gapped DNA substrates, which is experimentally observed (19, 20), can be realized in both the (pol β)$_{16}$ and (pol β)$_{5}$ binding modes. Such a distributive synthesis would result from the fact that the 31-kDa domain is involved in the interactions with the ssDNA in the (pol β)$_{16}$ mode. After every catalytic step, there will be an equilibrium between the complex where the 31-kDa domain is associated with a primer and the complex where the domain is weakly associated with the available ssDNA template, thus decreasing the probability of continuing the DNA synthesis.

The existence of the low site size binding mode may provide

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**TABLE II**

| Parameter | NaCl (mM) |
|-----------|-----------|
|           | 50        | 75        | 100       | 150       |
| $K_v$ (M$^{-1}$) | $(7 \pm 3) \times 10^6$ | $(1 \pm 0.1) \times 10^6$ | $(7 \pm 3) \times 10^6$ | $(8 \pm 4) \times 10^4$ |
| $K_v$ (M$^{-1}$) | $(1 \pm 0.0) \times 10^6$ | $(3 \pm 1.3) \times 10^4$ | $(2 \pm 1) \times 10^4$ | $(1 \pm 0.5) \times 10^4$ |
| $\Delta F_{\text{max}}$ (pol β)$_{15}$ | 15 ± 6 | 14 ± 6 | 15 ± 6 | 15 ± 3 |
| $\Delta F_{\text{max}}$ (pol β)$_{5}$ | 0.6 ± 0.1 | 0.5 ± 0.1 | 0.5 ± 0.1 | 0.3 ± 0.1 |
| $\Delta F_{\text{max}}$ (pol β)$_{5}$ | 1.2 ± 0.1 | 1.1 ± 0.1 | 1.0 ± 0.1 | 0.5 ± 0.1 |

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$^2$ Jezewiska, M. J., Rajendran, S., and Bujalowski, W. (1998) J. Mol. Biol., in press.
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TABLE III

| Parameter | poly(dA) | poly(dA) | poly(dT) | poly(dC) |
|-----------|----------|----------|----------|----------|
| $K_d$ (M$^{-1}$) | (1 ± 0.5) × 10$^5$ | (3 ± 1.5) × 10$^3$ | (1 ± 0.5) × 10$^5$ | (6 ± 3) × 10$^4$ |
| $K_a$ | 1 ± 0.5 | 1 ± 0.5 | 1 ± 0.5 | 1 ± 0.5 |
| $K_c$ (M$^{-1}$) | (5.5 ± 2.5) × 10$^4$ | (1 ± 0.5) × 10$^3$ | (2 ± 1) × 10$^4$ | (7 ± 3) × 10$^3$ |
| $K_a_2$ | 3 ± 1.5 | 3 ± 2 | 3 ± 2 | 3 ± 2 |
| $K_a_3$ | 4 ± 2 | 1 ± 0.5 | 1 ± 0.5 | 1 ± 0.5 |

The thermodynamic parameters for the nonfluorescent polymers have been determined by the MCT method using the oligomer dA(pA)$_n$ as a reference fluorescent nucleic acid (see "Experimental Procedures; Ref. 29). The errors are standard deviations determined using four to five independent titration curves.

an important insight as to how pol β is able to efficiently recognize the small ssDNA gaps of 3–5 residues and how the polymerase may adjust to the diminishing size of the gap. The previous model, based on studies of the analogous rat pol β binding to poly(rA), assumes a single binding mode of the enzyme-ssDNA complex with the ~11-nucleotide site size much longer than the site size of <6 nucleotides of the ssDNA gap in the physiological substrates of the enzyme (2, 3, 5, 19). The existence of the low site size binding mode indicates that the enzyme can efficiently recognize and associate with the ssDNA in the 3–5-nucleotide gap, which is well below the previously determined site size of the analogous rat pol β-poly(rA) complex (18). Such initial, efficient recognition of the small 3–5-nucleotide gaps would be predominantly based on the high affinity of the enzyme’s 8-kDa domain for the ssDNA, although the formation of the final specific complex will certainly include the interactions with adjacent dsDNA (4, 19, 20). The transition from the (pol β)$_{15}$ to the (pol β)$_{2}$ mode, as a result of the decreasing availability of the ssDNA, also indicates that pol β has the ability to assume a low site size binding mode, as the size of the gap decreases during DNA synthesis.

It should be pointed out that the site size of 5 nucleotide residues is still higher than the smallest gaps of 1–2 nucleotides proposed to be specifically recognized by an analogous rat pol β enzyme (19). On the other hand, the mechanism of the initial recognition of such small ssDNA gaps, in principle, could be different from the mechanism of the recognition of the larger 3–5-nucleotide gaps. Because of the overwhelming presence of the dsDNA, the mechanism of the recognition of 1–2 nucleotide gaps most probably includes both ss and dsDNA binding sites of the polymerase, even at the initial stages of the gap recognition process. In the absence of the ssDNA, the binding efficiency of the enzyme is mainly determined not by the high ssDNA affinity of the 8-kDa domain, but by the significantly lower dsDNA affinity, located on the 31-kDa domain of the enzyme. Experimental evidence supporting this hypothesis already exists. The affinity of the analogous enzyme toward 1–2 nucleotide gaps is significantly lower as compared with its binding to 5 nucleotide ssDNA gaps. The role of the 8-kDa domain in the recognition process is evident by the fact that the gap recognition is amplified by the presence of the 5’-terminal phosphate in the gap, which is specifically recognized only by the 8-kDa domain (19). Our laboratory is currently examining these interactions.

Binding of Human pol β to the ssDNA Is Characterized by Very Low Cooperativity—An important feature of the interactions of human pol β with the ssDNA is the lack of significant cooperative interactions for all studied ssDNAs, indicating that cooperative interactions are independent of the type of the nucleic acid base (Tables I–III). Such behavior may reflect a fundamental aspect of the function of a DNA-repair polymerase whose physiological function is to perform DNA synthesis at the specific damaged site, with limited access to the ssDNA. Such a function does not require the formation of polymerase clusters and/or extensive coating of long stretches of the ssDNA by the protein. Thus, very weak, or nonexistent, cooperativity in human pol β binding to the ssDNA, as determined in this work, may prove to be a general property of DNA-repair polymerases to perform functions that require only a single enzyme molecule.

Relation to the Other Works on Mammalian pol β Interactions with the ss Nucleic Acids—As we mentioned above, rigorous, quantitative studies of interactions between human pol β and the ssDNA have not been previously reported. Previous estimates of the stoichiometries of a mammalian pol β-ss nucleic acid complex and the mechanism of binding were based on studies of the binding of an analogous rat pol β, with the fluorescent polyribonucleotide, poly(rA) (18). These previous investigations indicated the formation of a single type of intact rat pol β complex with poly(rA), with ~11 nucleotide residues occluded by the enzyme (18). The existence of two binding modes in the interactions of the intact polymerase with poly(rA) was not detected. As a result, the binding isotherms were analyzed using the single binding mode, the McGhee-von Hippel model, instead of the model of the two binding modes described here.

Human and rat pol β are different analogous enzymes, and it is a natural expectation that the major features of their interactions with ssDNA will be similar, although such an expectation must always be experimentally tested. On the other hand, we believe that the main discrepancies between our data and these previous studies result from the fact that the quantitative approach used in our work has not been systematically applied in the previous investigations (18). Additionally, the discrepancies may be the result of using polyribonucleotide (poly(rA)) in these earlier studies and/or the different solution conditions applied, which could affect the mutual distribution of both binding modes.

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