Interactions of *Escherichia coli* Primary Replicative Helicase DnaB Protein with Nucleotide Cofactors*

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ABSTRACT Interactions between the *Escherichia coli* primary replicative helicase DnaB protein and nucleotide cofactors have been studied using several fluorescent nucleotide analogs and unmodified nucleotides. The thermodynamically rigorous fluorescent titration technique has been used to obtain true binding isotherms, independently of the assumptions of any relationships between the observed quenching of protein fluorescence and the degree of nucleotide binding. Fluorescence titrations using several MANT derivatives of nucleoside diphosphates (MANT-ADP, 3′,2′-*O*-(*N*-methylantraniloyl)adenosine-5′-diphosphate; MANT-GDP, 3′,2′-*O*-(*N*-methylantraniloyl)guanosine-5′-diphosphate; MANT-CDP, 3′,2′-*O*-(*N*-methylantraniloyl)cytidine-5′-diphosphate; MANT-UDP, 3′,2′-*O*-(*N*-methylantraniloyl)uridine-5′-diphosphate) have shown that the DnaB helicase has a preference for purine nucleotides. Binding of all modified nucleotides is characterized by similar negative cooperativity, indicating that negative cooperative interactions are base-independent. Thermodynamic parameters for the interactions of the unmodified nucleotides (ADP, GDP, CDP, and UDP) and inorganic phosphate (P_i) have been obtained by using the competition titration approach. To analyze multiple ligand binding to a finite circular lattice, for a general case in which each lattice binding site can exist in different multiple states, we developed a matrix method approach to derive analytical expressions for the partition function and the average degree of binding for such cases. Application of the theory to competition titrations has allowed us to extract the intrinsic binding constants and cooperativity parameters for all unmodified ligands. This is the first quantitative estimate of affinities and the mechanism of binding of different unmodified nucleotides and inorganic phosphate for a hexameric helicase. The intrinsic affinities of all of the studied ATP analogs are lower than the intrinsic affinities of the corresponding ADP analogs. The implications of these results for the mechanism of helicase action are discussed.

INTRODUCTION

The DnaB protein is a crucial replication protein in *Escherichia coli* involved both in initiation and elongation stages of DNA replication (McMacken et al., 1977; Ueda et al., 1978; Matson and Kaiser-Rogers, 1990; Kornberg and Baker, 1992). The protein is the *E. coli* primary replicative helicase, i.e., the factor responsible for the unwinding of the DNA duplex in front of the replication fork (LeBowitz and McMacken, 1986; Baker et al., 1987). DnaB protein is the only helicase required to reconstitute DNA replication in vitro from the chromosomal origin of replication (oriC). The DnaB protein exists in solution as a hexamer composed of six identical subunits (Reha-Krantz and Hurwitz, 1978; Bujalowski et al., 1994; Jezewska and Bujalowski, 1996a). Sedimentation equilibrium and sedimentation velocity studies show that the DnaB helicase exists as a stable hexamer in a large protein concentration range (Bujalowski et al., 1994). Magnesium ions play a crucial structural role in stabilizing the hexameric structure of the DnaB helicase. Hydrodynamic and EM data indicate that six protomers aggregate with cyclical symmetry in which the protomer-protomer contacts are limited to only two neighboring subunits (Bujalowski et al., 1994; San Martin et al., 1995; Yu et al., 1996).

Interactions of the DnaB helicase with ssDNA and the structure of the formed complexes have only recently been quantitatively studied (Bujalowski and Jezewska, 1995). On the basis of thermodynamically rigorous fluorescence titrations, we have established that the stoichiometry of the DnaB hexamer complex with the polymer ssDNA (site size) is 20 ± 3 nucleotides, and that the hexamer has only a single, strong ssDNA binding site. Moreover, photo-cross-linking experiments indicate that only a limited set of subunits, most probably only one, is engaged in the complex with the nucleic acid (Bujalowski and Jezewska, 1995; Jezewska and Bujalowski, 1996b; Jezewska et al., 1996). These data preclude any extensive wrapping of the nucleic acid around all six subunits of the hexamer; nor are they compatible with the models of the helicase translocation and

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Abbreviations used: EM, electron microscopy; TNP-ATP, 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; TNP-ADP, 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-diphosphate; ϵADP, 1,N^6-ethenoadenosine diphosphate; NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; MANT-AMP-PNP, 3′,2′-*O*-(*N*-methylantraniloyl)β,γ-imodoadenosine-5′-triphosphate; MANT-ADP, 3′,2′-*O*-(*N*-methylantraniloyl)adenosine-5′-diphosphate; MANT-GDP, 3′,2′-*O*-(*N*-methylantraniloyl)guanosine-5′-diphosphate; MANT-CDP, 3′,2′-*O*-(*N*-methylantraniloyl)cytidine-5′-diphosphate; P_i, inorganic phosphate; MANT-UDP, 3′,2′-*O*-(*N*-methylantraniloyl)uridine-5′-diphosphate; Tris, tris(hydroxymethyl) aminomethane; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); AMP-PNP, β,γ-imodoadenosine-5′-triphosphate.

*This work is dedicated to Prof. Serge N. Timasheff on his 70th birthday.

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unwinding dsDNA in which all subunits are simultaneously involved in the binding of the nucleic acid (Bujalowski and Jezewska, 1995; Jezewska et al., 1996).

The binding, or binding and hydrolysis, of ATP is the key element regulating DnaB protein activities (Arai and Kornberg, 1981a,b; Reha-Krantz and Hurwitz, 1978; LeBowitz and McMacken, 1986). Quantitative studies of the nucleotide binding to the DnaB helicase have established that the hexamer has six nucleotide binding sites, presumably one on each protomer (Arai and Kornberg, 1981b; Bujalowski and Klonowska, 1993). We have determined that the binding process is biphasic, resulting from the negative cooperative interactions among binding sites. The statistical thermodynamic model (the hexagon), in which the negative cooperativity is limited to neighboring subunits, provides an excellent description of the binding process of nucleotides to the DnaB helicase (Bujalowski and Klonowska, 1993, 1994a,b).

Early steady-state enzyme kinetic studies suggested that the hydrolysis of nucleoside triphosphates, as well as the helicase activity, is relatively independent of the nature of the base (Arai and Kornberg, 1981b; LeBowitz and McMacken, 1986). On the other hand, the DnaB helicase could not hydrolyze either deoxynucleoside triphosphates or TNP-ATP with the TNP fluorescent group attached to the 2′ and 3′ oxygens of the ribose (Bujalowski and Klonowska, 1993; Biswas et al., 1986). Moreover, the enzyme could not unwind duplex DNA in the presence of only deoxynucleotides, indicating intimate involvement of the sugar moiety of the nucleotide in the enzyme catalysis (LeBowitz and McMacken, 1986). Both γ-phosphate and intact ribose, but not the base moiety, appeared to be decisive elements in inducing allosteric interactions between the nucleotide and the ssDNA binding site (Jezewska and Bujalowski, 1996a). In the presence of AMP-PNP, the affinity of the enzyme toward ssDNA increases by ~3-4 orders of magnitude when compared to the affinity in the absence of any nucleotide or in the presence of ADP, respectively (Jezewska and Bujalowski, 1996a). The data suggest that different structural regions of the bound nucleotide molecule seem to be able to act independently, triggering specific responses of the DnaB helicase through highly localized conformational changes (Bujalowski and Klonowska, 1994a,b).

Understanding the energetics and kinetics of the interactions of the DnaB protein with nucleotide cofactors is indispensable for understanding different activities of the enzyme. This knowledge is a prerequisite for formulating any model of the mechanism of enzyme functioning in DNA replication, including translocation on the nucleic acid lattice and the catalysis of nucleic acid unwinding.

In this communication we examine interactions between the *E. coli* primary replicative helicase DnaB protein with several fluorescent nucleotide analogs and unmodified nucleotides by using the thermodynamically rigorous fluorescent titration technique. A general matrix method is developed to derive analytical expressions for the partition function and the average degree of binding; which describe multiple states, multiple ligand binding to a finite circular lattice. Application of the theory to competition titrations allowed us to extract the intrinsic binding constants and cooperativity parameters for all unmodified nucleotides (ADP, GDP, CDP, and UDP) and inorganic phosphate (P_i). The obtained data suggest that the hexameric helicase acquires free energy transducing capabilities when complexed with ssDNA, thus forming a "holoenzyme."

MATERIALS AND METHODS

Reagents and buffers

All chemicals were reagent grade. All solutions were made with distilled and deionized 18 MΩ (Mili-Q) water. The standard buffer (T2) is 50 mM Tris adjusted to pH 8.1 at appropriate temperatures with HCl, 5 mM $MgCl_2$, 10% glycerol. All fluorescent nucleotides have been prepared as previously described (Bujalowski and Klonowska, 1994a,b).

DnaB protein

The *E. coli* DnaB protein was purified as described by Bujalowski and Klonowska (1993). The concentration of the protein was determined spectrophotometrically, using the extinction coefficient $\epsilon_{280} = 1.85 \times 10^5\ cm^{-1}\ M^{-1}$ (hexamer) (Bujalowski et al., 1994; Jezewska et al., 1996).

Fluorescence measurements

All titrations of the DnaB protein with fluorescent nucleotide analogs were performed using a SLM 48000S spectrofluorometer (Spectronics, Rochester, NY). To avoid possible artifacts due to the fluorescence anisotropy of the sample, polarizers were placed in excitation and emission channels and set at 90° and 55° (magic angle), respectively. The binding was followed by monitoring the quenching of the protein tryptophan fluorescence (λ_{ex} = 300 nm, λ_{em} = 345 nm). All titration points were corrected for dilution and inner filter effects using the following formula (Parker, 1968):

$$F_{icor} = (F_i - B_i)\left(\frac{V_i}{V_o}\right)10^{0.5b(A_{i\lambda ex} + A_{i\lambda em})} \quad (1)$$

where F_{icor} is the corrected value of the fluorescence intensity at a given point of titration i, F_i is the experimentally measured fluorescence intensity, B_i is the background, V_i is the volume of the sample at a given titration point, V_o is the initial volume of the sample, b is the total length of the optical path in the cuvette expressed in centimeters, and $A_{i\lambda ex}$ and $A_{i\lambda em}$ are the absorbances of the sample at excitation and emission wavelengths, respectively. Nonlinear least-squares fits of binding isotherms and computer simulations were performed using Mathematica software (Wolfram Research, Champaign, IL).

Determination of rigorous thermodynamic binding isotherms

In the studies described in this work, we followed the binding of the nucleotides to the DnaB protein by monitoring the quenching of the protein fluorescence, ΔQ_{obsd}, upon the complex formation. A general procedure to obtain absolute estimates of the average degree of binding of the nucleotide, $\Sigma\nu_i$, and the free nucleotide concentration, N_F, has previously been described (Bujalowski and Klonowska, 1993, 1994a,b). Briefly, if there are i different complexes of DnaB hexamer with a nucleotide cofactor, then the experimentally observed ΔQ_{obsd} is functionally related to $\Sigma\nu_i$ by Eq. 2 (Bujalowski and Klonowska, 1993; Jezewska and Bujalowski, 1996b),

$$\Delta Q_{obsd} = \sum \nu_i \Delta Q_{imax}, \tag{2}$$

where ΔQ_{imax} is the maximum quenching of the protein fluorescence in complex i. Thus, ΔQ_{obsd} is the degree of binding, $\Sigma\nu_i$, weighted by the contributions to the overall fluorescence quenching from different complexes. Therefore, for the same value of ΔQ_{obsd} obtained at two different total protein concentrations, P_{T1} and P_{T2}, the degree of binding, $\Sigma\nu_i$, and the free nucleotide concentration, N_F, must also be the same. The values of $\Sigma\nu_i$ and N_F are then related to the total protein concentrations, P_{T1} and P_{T2}, and the total nucleotide concentrations, N_{T1} and N_{T2}, at which the same ΔQ_{obsd} is obtained by the formulas

$$\sum \nu_i = \left(\frac{N_{T2} - N_{T1}}{P_{T2} - P_{T1}}\right) \tag{3a}$$

$$N_F = N_{TX} - \sum \nu_i(P_{TX}), \tag{3b}$$

where $x = 1$ or 2 (Bujalowski and Klonowska, 1993).

In the case of the DnaB protein, which has six nucleotide-binding sites, it is practically impossible to obtain independently all optical parameters (ΔQ_{imax}) for different complexes. This problem can be solved by introducing a representation of the observed fluorescence quenching as a function of the nucleotide binding through an empirical function that makes possible the analysis of single binding isotherms without the necessity of determining all quenching constants for different complexes. An empirical function, usually polynomial, is found that relates the experimentally determined dependence of the average quenching, ΔQ_{obsd}, to $\Sigma\nu_i$. We achieved this by using a nonlinear least-squares fit of ΔQ_{obsd} as a function of $\Sigma\nu_i$. In the case of DnaB-nucleotide interactions, a minimum third-degree polynomial was necessary to describe this function, as defined by

$$\Delta Q_{obsd} = a(\sum \nu_i) + b(\sum \nu_i)^2 + c(\sum \nu_i)^3, \tag{4}$$

where a, b, and c are fitting constants. This function was then used to generate a theoretical fluorescence isotherm for a binding model and to extract true binding parameters from the experimentally obtained single isotherm (Bujalowski and Klonowska, 1993).

RESULTS

Analytical expressions for the partition function and degree of binding for multiple state, multiple ligand binding to a finite circular lattice

The DnaB hexamer binds six nucleotide molecules at saturation, presumably one on each subunit. Moreover, the binding process is characterized by negative cooperativity between neighboring subunits of the hexamer (Bujalowski and Klonowska, 1993). Hydrodynamic and EM data show that, in the hexamer, the protomers form a cyclic, ringlike structure with physical contacts between subunits limited to two neighbors (Bujalowski et al., 1994; San Martin et al., 1995; Yu et al., 1996; Fig. 1). From the standpoint of statistical thermodynamics, binding of the nucleotide molecules to the DnaB hexamer can be described using a finite circular lattice model (Hill, 1985). In the simplest case, each subunit of the hexamer can exist in only two states, free and bound with a nucleotide molecule. The analytical expressions for the partition function and the average degree of binding, $\Sigma\nu_i$, for the DnaB hexamer-nucleotide system can then be obtained using the matrix method (Hill, 1985). We assigned a statistical weight for the free sites as 1, and the statistical weight for a site bound with the nucleotide as KL, where K is the intrinsic binding constant and L is the free nucleotide concentration. The transition matrix $\mathbf{M}_A$,

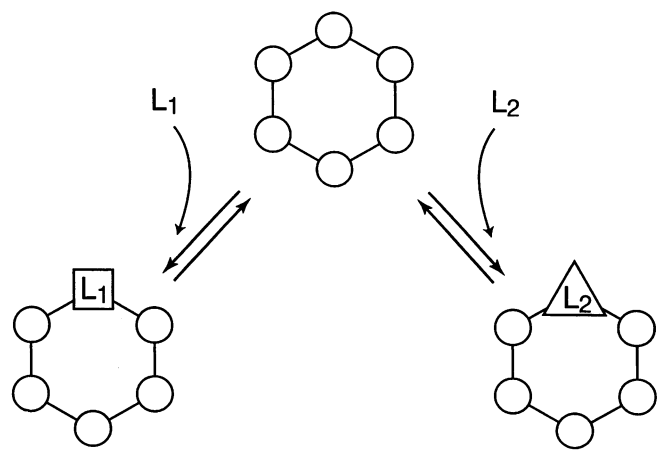

FIGURE 1 A schematic representation of the ringlike structure of the DnaB protein hexamer (hexagon) and the possible three states (○, □, △) of the single binding site of the hexamer in the competition titration studies described in this work. The circles represent subunits and the lines connecting each subunit with its neighbors symbolize the number of possible cooperative interactions (two in the hexagon model). L_1 and L_2 symbolize the reference fluorescent nucleotide and the unmodified nucleotide, respectively (see text for details).

which correlates the states of neighboring subunits, is then defined as

$$\mathbf{M}_A = \begin{pmatrix} 1 & KL \\ 1 & \sigma KL \end{pmatrix}, \tag{5}$$

where the parameter σ characterizes the cooperative interactions between neighboring subunits, both bound with the nucleotide molecule.

The partition function of this two-state macromolecule-ligand system can be formulated by first obtaining the eigenvalues λ of the transition matrix $\mathbf{M}_A$, which is accomplished by solving the characteristic equation

$$\begin{vmatrix} 1-\lambda & KL \\ 1 & \sigma KL - \lambda \end{vmatrix} = 0 \tag{6}$$

with

$$\lambda_1, \lambda_2 = \frac{1 + \sigma KL \pm \sqrt{(1-\sigma KL)^2 + 4KL}}{2}. \tag{7}$$

For the circular lattice, the partition function Z_A is then related to the eigen values by the relationship (Thompson, 1968; Hill, 1985)

$$Z_A = \lambda_1^n + \lambda_2^n, \tag{8}$$

where n is the number of binding sites on the macromolecule.

The average degree of binding is then defined using the standard statistical thermodynamic formula (Thompson, 1968; Hill, 1985)

$$\sum \nu_i = \frac{\partial \ln Z_A}{\partial \ln L}. \tag{9}$$

Inspection of Eqs. 5–9 shows that analytical expressions for eigenvalues, partition function, and the degree of binding can only be obtained in the simplest case, where each subunit (binding site) can only exist in two states, free and bound. In such cases, the characteristic equation of the transition matrix is a quadratic polynomial. On the other hand, if the binding site can exist in more than two states, the characteristic equation becomes a polynomial of orders higher than 2 and the analytical expressions for the eigenvalues are very difficult, if not impossible, to obtain. In such situations one usually has to resort to complex numerical calculations. However, we will show below that the analytical expressions for the partition function and the degree of ligand binding can be derived for the case where the binding site can exist in an arbitrary number of states.

The partition function of a circular lattice of n discrete binding sites can be expressed as a trace of the matrix, where $\mathbf{M}_G$ is the transition matrix of the generalized system (Hill, 1985)

$$Z_G = T_r(\mathbf{M}_G^n). \tag{10}$$

In a general case, where each binding site can exist in different p states, the transition matrix $\mathbf{M}_G$ is a $p \times p$ matrix and is defined as

$$\mathbf{M}_G = \begin{pmatrix} a_{11} & \cdot & \cdot & \cdot & \cdot & \cdot & a_{1p} \\ \cdot & \cdot & & & & & \cdot \\ \cdot & & \cdot & & & & \cdot \\ \cdot & & & \cdot & & & \cdot \\ \cdot & & & & \cdot & & \cdot \\ \cdot & & & & & \cdot & \cdot \\ a_{p1} & \cdot & \cdot & \cdot & \cdot & \cdot & a_{pp} \end{pmatrix}, \tag{11}$$

where the elements, a, are the statistical weights of different states of a binding site correlated with the states of the neighboring site (see Eq. 5).

The trace of a matrix is the sum of its diagonal elements (Hill, 1985). On the other hand, each diagonal element of the matrix can be expressed using the original transition matrix $\mathbf{M}_G$ of statistical weights. The first term in the trace is then

$$T(1,1) = (10..000) \cdot \begin{pmatrix} a_{11} & \cdot & \cdot & \cdot & \cdot & \cdot & a_{1p} \\ \cdot & \cdot & & & & & \cdot \\ \cdot & & \cdot & & & & \cdot \\ \cdot & & & \cdot & & & \cdot \\ \cdot & & & & \cdot & & \cdot \\ \cdot & & & & & \cdot & \cdot \\ a_{p1} & \cdot & \cdot & \cdot & \cdot & \cdot & a_{pp} \end{pmatrix}^n \begin{pmatrix} 1 \\ 0 \\ \cdot \\ \cdot \\ 0 \\ 0 \\ 0 \end{pmatrix}. \tag{12}$$

Any (j, j) term in the trace can be defined as

$$T(j,j) = (00.1_j.00) \begin{pmatrix} a_{11} & \cdot & \cdot & \cdot & \cdot & \cdot & a_{1p} \\ \cdot & \cdot & & & & & \cdot \\ \cdot & & \cdot & & & & \cdot \\ \cdot & & & \cdot & & & \cdot \\ \cdot & & & & \cdot & & \cdot \\ \cdot & & & & & \cdot & \cdot \\ a_{p1} & \cdot & \cdot & \cdot & \cdot & \cdot & a_{pp} \end{pmatrix}^n \begin{pmatrix} 0 \\ 0 \\ \cdot \\ 1_j \\ \cdot \\ 0 \\ 0 \end{pmatrix}. \tag{13}$$

Thus, the last term in the trace is

$$T(p,p) = 00..001) \begin{pmatrix} a_{11} & \cdot & \cdot & \cdot & \cdot & \cdot & a_{1p} \\ \cdot & \cdot & & & & & \cdot \\ \cdot & & \cdot & & & & \cdot \\ \cdot & & & \cdot & & & \cdot \\ \cdot & & & & \cdot & & \cdot \\ \cdot & & & & & \cdot & \cdot \\ a_{p1} & \cdot & \cdot & \cdot & \cdot & \cdot & a_{pp} \end{pmatrix}^n \begin{pmatrix} 0 \\ 0 \\ \cdot \\ \cdot \\ 0 \\ 0 \\ 1 \end{pmatrix}. \tag{14}$$

Therefore

$$Z_G = Tr\mathbf{M}_G^n = \sum_{j=1}^{p} T(j,j). \tag{15}$$

Eq. 15 is the desired general analytical expression for the partition function for the macromolecule-ligand system

where each binding site can exist in an arbitrary p number of states. The analytical equation for the average number of subunits in a given state p, ν_p, can then be found by applying the relationship analogous to Eq. 9,

$$\nu_p = \frac{\partial \ln Z_G}{\partial \ln s}, \tag{16}$$

where s is any factor in the partition function Z_G, which is in a one-to-one correspondence with a given p state. Expanding Eqs. 15 and 16 provides higher degree, simple polynomials (although they are large for high values of p and n). Application of Eqs. 10–16 completely eliminates the necessity of solving pth degree characteristic equations in the case of ligand binding to a multiple-state lattice, which is not possible in a general case for $p > 2$. It should be pointed out that the extension of this analytical approach to more complex models, including conformational transitions accompanying the binding, is straightforward.

In the experiments presented below, we examined the binding of the reference fluorescent derivative of a nucleotide to the six binding sites of the DnaB hexamer in the presence of different concentrations of competing unmodified nucleotides. This is the situation where each binding site of the DnaB hexamer can exist in three different states, free, bound with fluorescent nucleotide, and bound with the unmodified nucleotide. The transition matrix of statistical weights, $\mathbf{M}_T$, for this three-state binding model is defined as

$$\mathbf{M}_T = \begin{pmatrix} 1 & K_1L_1 & K_2L_2 \\ 1 & \sigma_1K_1L_1 & \sigma_3K_2L_2 \\ 1 & \sigma_3K_1L_1 & \sigma_2K_2L_2 \end{pmatrix} \tag{17}$$

where K_1 is the intrinsic binding constant of the fluorescent reference nucleotide, L_1 is the free concentration of the fluorescent nucleotide, σ_1 is the parameter characterizing cooperative interactions between bound fluorescent nucleotides, K_2 is the intrinsic binding constant of the unmodified nonfluorescent nucleotide, L_2 is the free concentration of the unmodified nucleotide, σ_2 is the parameter characterizing cooperative interactions between unmodified nucleotides, and σ_3 is the parameter characterizing cooperative interactions between fluorescent and unmodified nucleotides. Analogously to Eq. 15, the partition function Z_T for the binding of the fluorescent nucleotide in the presence of a nonfluorescent, unmodified nucleotide is defined as

$$Z_T = (100)\mathbf{M}_T^6\begin{pmatrix} 1 \\ 0 \\ 0 \end{pmatrix} + (010)\mathbf{M}_T^6\begin{pmatrix} 0 \\ 1 \\ 0 \end{pmatrix} + (010)\mathbf{M}_T^6\begin{pmatrix} 0 \\ 1 \\ 0 \end{pmatrix}, \tag{18}$$

where the three matrix terms in Eq. 18 are the terms in the trace of the matrix.

The quantity that is experimentally determined, the average degree of binding, $\Sigma\nu_i$, of the fluorescent nucleotide, is then

$$\sum \nu_i = \frac{\partial \ln Z_T}{\partial \ln L_1} \tag{19a}$$

and

$$\sum \nu_i = \frac{(100)\left[\frac{\partial(\mathbf{M}_T^6)}{\partial \ln L_1}\right]\begin{pmatrix} 1 \\ 0 \\ 0 \end{pmatrix} + (010)\left[\frac{\partial(\mathbf{M}_T^6)}{\partial \ln L_1}\right]\begin{pmatrix} 0 \\ 1 \\ 0 \end{pmatrix} + (001)\left[\frac{\partial(\mathbf{M}_T^6)}{\partial \ln L_1}\right]\begin{pmatrix} 0 \\ 0 \\ 1 \end{pmatrix}}{Z_T}, \tag{19b}$$

where $\mathbf{M}_T$ is defined by Eq. 17. Expressions 17–19 contain five interaction parameters, K_1, σ_1, K_2, σ_2, and σ_3. However, notice that K_1 and σ_1 can be determined independently from the fluorescence titration of the DnaB hexamer with a fluorescent nucleotide analog in the absence of a competing, unmodified nucleotide. Therefore, there are only three parameters, K_2, σ_2, and σ_3, which can be extracted by nonlinear least-squares fits of the experimental binding isotherms.

Binding of ATP and ADP fluorescent analogs to the DnaB hexamer

The interactions of ATP and ADP analogs with the DnaB helicase have been performed with nucleotide derivatives bearing the fluorescent modification in different locations on the nucleotide. The MANT group is located on the ribose, and the etheno modification is located on the base (Bujalowski and Klonowska, 1993, 1994a,b). Fluorescence titrations of the DnaB helicase with MANT-AMP-PNP and MANT-ADP in buffer T2 (pH 8.1 20 mM NaCl, 20°C) are shown in Fig. 2 *a*. The solid lines are computer fits of the binding isotherms to the hexagon model (Eqs. 5–9; Bujalowski and Klonowska, 1993). Although the negative cooperativity is similar in the case of binding ATP and ADP analogs, the intrinsic affinity of MANT-ADP ($K = (9 \pm 3) \times 10^5\ M^{-1}$) is higher by a factor of ~2 compared to the affinity of the MANT-AMP-PNP ($K = (5.5 \pm 1.5) \times 10^5\ M^{-1}$). The difference between the affinities of ATP and ADP analogs is even more pronounced in the case of base-modified etheno derivatives. Fluorescence titrations of the DnaB helicase with ϵAMP-PNP and ϵADP in buffer T2 (pH 8.1, 20 mM NaCl, 20°C) are shown in Fig. 2 *b*. As in the case of MANT analogs, the negative cooperativity factor, σ, is similar for ϵADP and ϵAMP-PNP. However, the intrinsic binding constant for ϵADP is ~7-fold higher ($K = (4 \pm 1) \times 10^5\ M^{-1}$) compared with the intrinsic binding

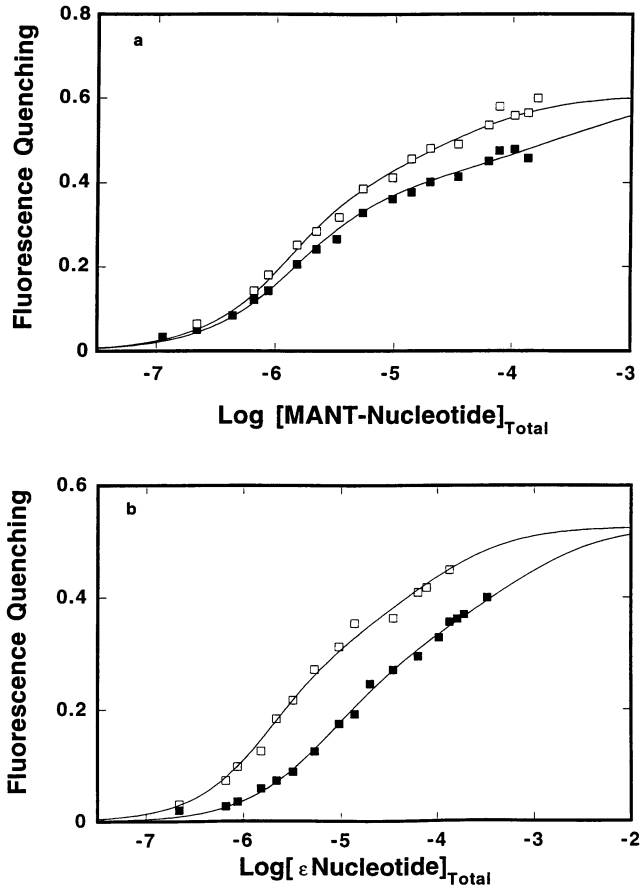

FIGURE 2 (*a*) Fluorescence titrations of the DnaB hexamer with MANT-AMP-PNP (■) and MANT-ADP (□) monitored by the quenching of the intrinsic protein fluorescence in buffer T2 (pH 8.1, 20 mM NaCl, 20°C). Solid lines are theoretical curves obtained by using a third-degree polynomial representing the exact relationship between the quenching of the protein fluorescence, Q, and the average degree of binding, $\Sigma\nu_i$ (Eq. 4 with $a = 18.33 \times 10^{-2}$, $b = -15.84 \times 10^{-4}$, $c = 3.679 \times 10^{-4}$) and interaction parameters (hexagon model, Bujalowski and Klonowska, 1993); MANT-AMP-PNP, $K = 5.5 \times 10^5\ M^{-1}$, $\sigma = 0.07$; MANT-ADP, $K = 9 \times 10^5\ M^{-1}$, $\sigma = 0.17$. (*b*) Fluorescence titration of the DnaB hexamer with ϵAMP-PNP (■) and ϵADP (□) monitored by the quenching of the intrinsic protein fluorescence in buffer T2 (pH 8.1, 20 mM NaCl, 20°C). Solid lines are theoretical curves obtained using a third-degree polynomial representing the exact relationship between the quenching of the protein fluorescence, Q, and the average degree of binding, $\Sigma\nu_i$ (Eq. 4 with $a = 14.1 \times 10^{-2}$, $b = -13.0186 \times 10^{-4}$, $c = 6.899 \times 10^{-4}$) and interaction parameters (hexagon model); ϵAMP-PNP, $K = 5.9 \times 10^4\ M^{-1}$, $\sigma = 0.2$; ϵADP, $K = 4 \times 10^5\ M^{-1}$, $\sigma = 0.22$. The DnaB protein concentration is 5.5×10^{-7} M (hexamer).

constant of ϵAMP-PNP ($K = (5.9 \pm 2) \times 10^4\ M^{-1}$). We have previously reported that the ribose-modified ATP analog, TNP-ATP, binds to the DnaB hexamer with an intrinsic affinity ~4-fold lower than that of TNP-ADP (Bujalowski and Klonowska, 1993). Thus, the higher affinity of the ADP analogs for the DnaB hexamer, as compared to the ATP analogs, is independent of fluorescent modification and is an intrinsic property of the DnaB helicase (see Discussion).

Binding of deoxyribonucleotides to the DnaB hexamer

Fluorescence titrations of the DnaB helicase with MANT-dATP and MANT-dADP in buffer T2 (pH 8.1, 20 mM NaCl, 20°C) are shown in Fig. 3, *a* and *b*. For comparison, the fluorescence titrations of the helicase with MANT-AMP-PNP and MANT-ADP are also included. The data show that the intrinsic affinity of deoxynucleotides is very

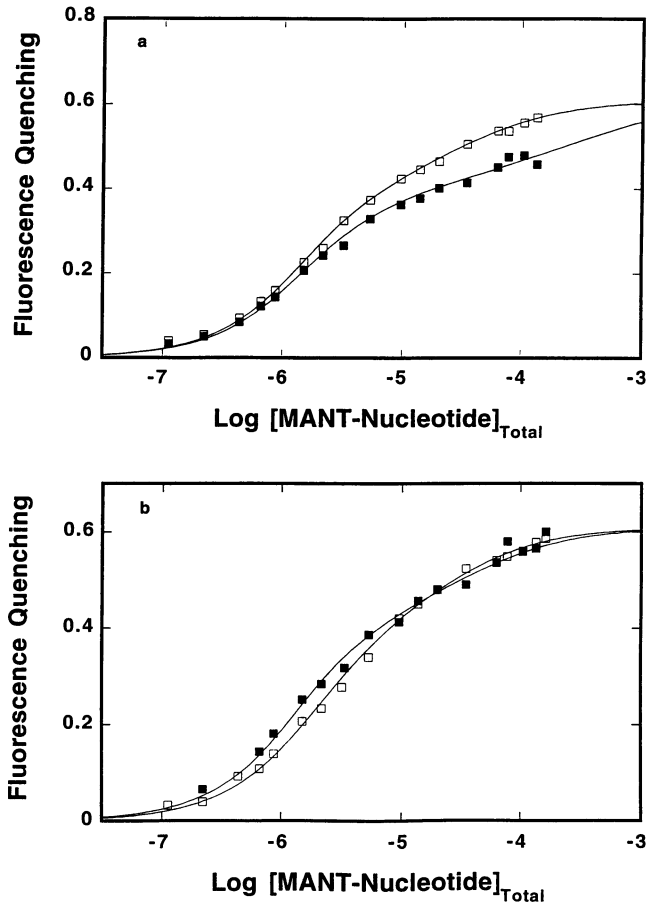

FIGURE 3 (*a*) Fluorescence titration of the DnaB hexamer with MANT-dATP (□) monitored by the quenching of the intrinsic protein fluorescence in buffer T2 (pH 8.1, 20 mM NaCl, 20°C). For comparison the titration curve of the DnaB helicase with MANT-AMP-PNP is also included (■) (data from Fig. 1). The solid line is a theoretical curve obtained using a third-degree polynomial representing the exact relationship between the quenching of the protein fluorescence, Q, and the average degree of binding, $\Sigma\nu_i$ (Eq. 4 with $a = 18.33 \times 10^{-2}$, $b = -15.84 \times 10^{-4}$, $c = 3.679 \times 10^{-4}$) and interaction parameters (hexagon model) $K = 7 \times 10^5\ M^{-1}$, $\sigma = 0.21$. (*b*) Fluorescence titration of the DnaB hexamer with MANT-dADP (□) monitored by the quenching of the intrinsic protein fluorescence in buffer T2 (pH 8.1, 20 mM NaCl, 20°C). For comparison, the titration curve of the DnaB helicase with MANT-ADP is also included (■) (data from Fig. 1). The solid line is a theoretical curve obtained using a third-degree polynomial representing the exact relationship between the quenching of the protein fluorescence, Q, and the average degree of binding, $\Sigma\nu_i$ (Eq. 4 with $a = 14.1 \times 10^{-2}$, $b = -13.0186 \times 10^{-4}$, $c = 6.899 \times 10^{-4}$) and interaction parameters (hexagon model); MANT-dADP, $K = 4.2 \times 10^5\ M^{-1}$, $\sigma = 0.3$. The DnaB protein concentration is 5.5×10^{-7} M (hexamer).

similar to the intrinsic affinity of the corresponding ribonucleotides. Thus, the lack of 2′ oxygen on the ribose does not significantly affect the intrinsic affinity of the nucleotide for its binding site on the DnaB helicase. However, contrary to the ribose analogs, MANT-dATP shows a slightly higher intrinsic affinity, characterized by $K = (7 \pm 1) \times 10^5\ M^{-1}$, as compared to MANT-dADP, which has $K = (4.2 \pm 0.5) \times 10^5\ M^{-1}$ (Fig. 3, *a* and *b*). Furthermore, the negative cooperativity in the binding of deoxynucleotides is lower than that of their ribose counterparts (MANT-AMP-PNP and MANT-ADP). This is particularly pronounced in the case of ATP analogs, with $\sigma = 0.07 \pm 0.03$ and 0.21 ± 0.05 characterizing the binding of MANT-AMP-PNP and MANT-dATP, respectively.

The hydrolysis of deoxyribonucleotides by the DnaB helicase is extremely inefficient (Arai and Kornberg, 1981b). Moreover, deoxyribonucleotides do not support the helicase activity of the enzyme and dATP does not affect the affinity of the helicase toward ss- or dsDNA (LeBowitz and McMacken, 1986; Jezewska and Bujalowski, 1996a; manuscript in preparation). It is evident that these dramatic differences between the ribose and deoxyribonucleotides, in affecting the enzyme action, do not result from lower affinities, but rather reflect subtle, specific interplays between the ribose and γ-phosphate that affect functional interactions in the nucleotide binding site of the helicase.

Base specificity in the nucleotide interactions with the DnaB helicase

Model-independent studies

Fluorescence titrations of the DnaB helicase with different nucleoside diphosphates, modified at the ribose with the same MANT group (MANT-ADP, MANT-GDP, MANT-CDP, and MANT-UDP) in buffer T2 (pH 8.1, 20 mM NaCl, 20°C) are shown in Fig. 4 *a*. Independent of the base type, the binding isotherms are biphasic, indicating the presence of negative cooperative interactions (Table 1). The purine nucleotides show higher affinities than the pyrimidine analogs. The MANT-GDP binding is characterized by the highest intrinsic binding constant ($K = (2 \pm 0.6) \times 10^6\ M^{-1}$). MANT-CDP shows the lowest affinity, with $K = (2.3 \pm 0.4) \times 10^5\ M^{-1}$.

Based on chemical studies, MANT-ATP and MANT-ADP were originally postulated to exist solely as 3′ isomers (Hiratsuka, 1983). On the other hand, NMR studies indicate that MANT-ATP and MANT-ADP may exist as an equilibrium mixture of 2′ and 3′ isomers (acyl migration), with the 3′ isomer predominating (Cremo et al., 1990). Because this equilibrium will affect all MANT derivatives, it should not affect the relative affinities. Nevertheless, to assess whether the MANT group changes the relative affinities of different nucleotides, we performed fluorescence titrations of the DnaB hexamer, with MANT-dADP as a reference fluorescent ligand, in the presence of competing, unmodified nucleotides (GDP, UDP, CDP, ADP) at the same concentration of each unmodified nucleotide (4×10^{-5} M) (Fig. 4 *b*). Because of the lack of 2′ oxygen, MANT-dADP is not subjected to 2′-3′ acyl migration. The titration curve of the DnaB protein with MANT-dADP shifts mostly toward higher MANT-dADP concentrations in the presence of GDP, indicating the highest affinity for this nucleotide, followed by ADP and UDP. The shift of the binding isotherm is least pronounced in the pres-

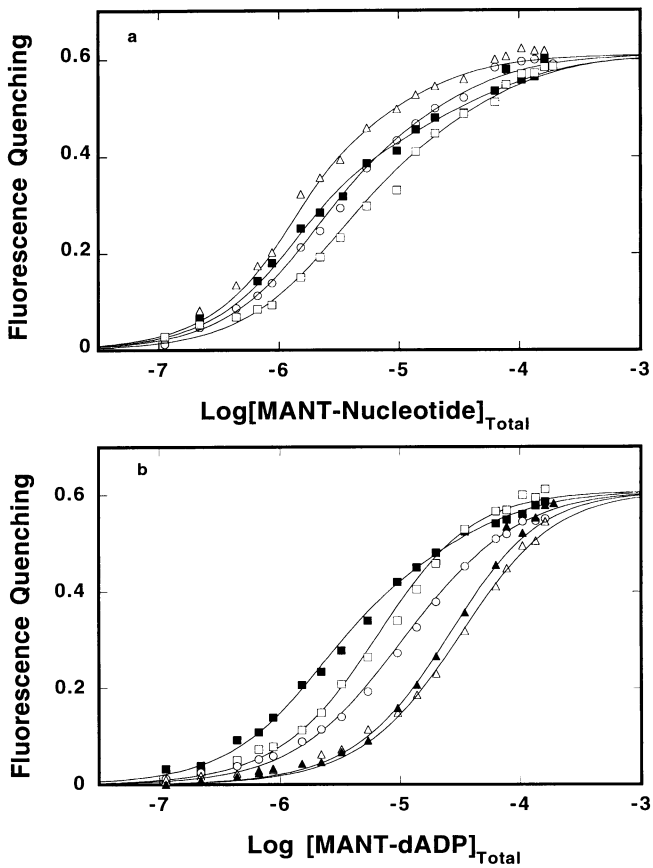

FIGURE 4 (*a*) Fluorescence titration of the DnaB hexamer with MANT derivatives of different nucleotides monitored by the quenching of the intrinsic protein fluorescence in buffer T2 (pH 8.1, 20 mM NaCl, 20°C). ■, MANT-ADP; △, MANT-GDP; □, MANT-CDP; ○, MANT-UDP. The solid lines are theoretical curves obtained by using a third-degree polynomial representing the empirical relationship between the quenching of the protein fluorescence, Q, and the average degree of binding, $\Sigma\nu_i$ (Eq. 4 with $a = 14.1 \times 10^{-2}$, $b = -13.0186 \times 10^{-4}$, $c = 6.899 \times 10^{-4}$) and interaction parameters (hexagon model). MANT-ADP, $K = 9 \times 10^5\ M^{-1}$, $\sigma = 0.17$; MANT-GDP, $K = 2 \times 10^6\ M^{-1}$, $\sigma = 0.25$; MANT-CDP, $K = 2.3 \times 10^5\ M^{-1}$, $\sigma = 0.33$; MANT-UDP, $K = 5 \times 10^5\ M^{-1}$, $\sigma = 0.33$. (*b*) Fluorescence titration of the DnaB hexamer with MANT-dADP monitored by the quenching of the intrinsic protein fluorescence, in buffer T2 (pH 8.1, 20 mM NaCl, 20°C), in the presence of the same concentration (5×10^{-5} M) of different unmodified nucleoside diphosphates. □, CDP; ○, UDP; ▲, ADP; △, GDP. For comparison, the titration curve with MANT-dADP alone is also included (■) (Fig. 3 *b*). The solid lines are computer fits obtained using a third-degree polynomial representing the exact relationship between the quenching of the protein fluorescence, Q, and the average degree of binding, $\Sigma\nu_i$ (Eq. 4 with $a = 14.1 \times 10^{-2}$, $b = -13.0186 \times 10^{-4}$, $c = 6.899 \times 10^{-4}$) and interaction parameters (hexagon model). $K = 1.1 \times 10^5\ M^{-1}$, $\sigma = 0.8$ (CDP); $K = 7 \times 10^4\ M^{-1}$, $\sigma = 0.6$ (UDP); $K = 2.4 \times 10^4\ M^{-1}$, $\sigma = 0.95$ (ADP); $K = 2 \times 10^5\ M^{-1}$, $\sigma = 0.85$ (GDP). The DnaB protein concentration is 5.5×10^{-7} M (hexamer).

TABLE 1 Intrinsic binding constant, K, and cooperativity parameter, σ, for the binding of different MANT-nucleoside diphosphates to the DnaB helicase in buffer T2 (pH 8.1, 20 mM NaCl, 20°C)

	MANT-ADP	MANT-GDP	MANT-UDP	MANT CDP
K (M^{-1})*	$(9 \pm 3) \times 10^5$	$(2 \pm 0.6) \times 10^6$	$(5 \pm 1) \times 10^5$	$(2.3 \pm 0.6) \times 10^5$
σ	0.17 ± 0.03	0.25 ± 0.03	0.33 ± 0.03	0.33 ± 0.03

The binding isotherms have been analyzed using the hexagon model (Bujalowski and Klonowska, 1993; see text for details).
* The errors associated with the determination of K and σ are standard deviations obtained from four to six repeated titration experiments.

ence of the competing CDP, indicating the lowest affinity of this nucleotide. Thus, relative affinities of different unmodified nucleotides are in excellent agreement with the results obtained from direct binding studies using different MANT derivatives (Fig. 4 *a*).

Competition binding studies establish whether different ligands bind to the same binding site. Fluorescence titrations of the DnaB helicase with MANT-dADP, in the presence of different concentrations of GDP, in buffer T2 (pH 8.1 20 mM NaCl, 20°C), are shown in Fig. 5 *a*. As the concentration of GDP increases, the titration curves are shifted toward a higher MANT-dADP concentration. Similar competition titration curves have been obtained for other nucleotides and inorganic phosphate (P_i), indicating strong competition between studied ligands and the reference MANT-dADP for the same binding site (data not shown).

The dependence of the intrinsic binding constants of MANT-dADP upon the logarithm of different nucleotides and inorganic phosphate concentrations is shown in Fig. 5 *b*. The plots are clearly not linear. Below a concentration of $\sim 5 \times 10^{-6}$ M, the slopes $\partial \log K/\partial \log[\text{Nucleotide}]$ approach ~ 0; however, at higher nucleotide concentrations, the intrinsic affinity decreases dramatically, indicating that all binding sites on the hexamer are initially predominantly saturated with competing nucleotide cofactors. In this concentration range, each [Nucleotide] $\gg$ $[\text{DnaB}]_{\text{total}}$, therefore, $[\text{Nucleotide}]_{\text{total}}$ is practically equal to $[\text{Nucleotide}]_{\text{free}}$. Thus, to determine the number of unmodified nucleotide molecules (GDP, ADP, UDP, CDP), m, competing with MANT-dADP for the binding site, one can use the linkage relation (Hill, 1985)

$$\frac{\partial \log K_{\text{MANT-dADP}}}{\partial \log[\text{Nucleotide}]} = -m. \qquad (20)$$

It should be pointed out that this is a general thermodynamic relation that is independent of any particular model for the unmodified nucleotide binding to the DnaB hexamer (see below). Analysis of the linear parts of the log-log plots for each studied nucleotide provides $\partial \log K_{\text{Mant-dADP}}/\partial \log[\text{Nucleotide}] = -0.85 \pm 0.1, -0.95 \pm 0.1, -0.85 \pm 0.1, -0.83 \pm 0.1$ for the ADP, GDP, UDP, and CDP, respectively. This indicates that a single molecule of each of the nucleotide cofactors competes with a single molecule of MANT-dADP for the same binding site. The log-log plot of the dependence of the intrinsic binding constant of the reference ligand MANT-dADP and inorganic phosphate concentrations shows the same characteristics as the log-log plot of all studied nucleoside diphosphates (Fig. 5 *b*). The plot is shifted toward higher concentrations because of the much lower affinity of inorganic phosphate, as compared to the nucleoside diphosphates. However, the linear part of the log-log plot at a high P_i concentration provides the value of $m = 0.95 \pm 0.1$, indicating that a single inorganic phosphate competes specifically with MANT-dADP for the phosphate binding site on the DnaB hexamer.

For comparison with nucleoside diphosphates and inorganic phosphate, we performed fluorescence titrations of the DnaB hexamer with MANT-dADP in the presence of different concentrations of NaBr. Sodium bromide was selected because Br^- has the highest nonspecific affinity among simple anions for anion-binding sites of the proteins (von Hippel and Schleich, 1969). Although the plot shows behavior similar to that of diphosphate ligands discussed above, it is shifted toward much higher concentrations, indicating much lower affinity of Br^- for the binding site. Moreover, the value of the slope $\partial \log K/\partial \log[\text{NaBr}] = -2.8 \pm 0.5$, which is much higher than the ~ 1 obtained for nucleoside diphosphates and P_i. Titrations with MANT-AMP-PNP, which has three phosphate groups, in the presence of different NaBr concentrations, show the same value of $\partial \log K/\partial \log[\text{NaBr}] = -2.8 \pm 0.5$, indicating that in the presence of NaBr, the number of ions released is independent of the number of phosphate groups on the nucleotide (data not shown). This high value of the slope, independent of the number of phosphate groups of the nucleotide cofactor, indicates a nonspecific ion (anion) release accompanying the binding of MANT-dADP and MANT-AMP-PNP rather than the 1:1 specific competition for the nucleotide binding site (see Discussion).

Determination of the intrinsic binding constants and cooperativity parameters for the unmodified nucleoside diphosphates, AMP, and inorganic phosphate binding to the DnaB protein hexamer

Analyses of the binding of the fluorescent nucleotide analogs to the DnaB helicase hexamer have been performed using a statistical thermodynamic model, the hexagon, which is a two-state binding model (Eqs. 5–9; Bujalowski and Klonowska, 1993). It provides an excellent description of the experimental binding isotherm, using only two parameters, the intrinsic binding constant, K, and the parameter σ, characterizing the cooperative interactions between

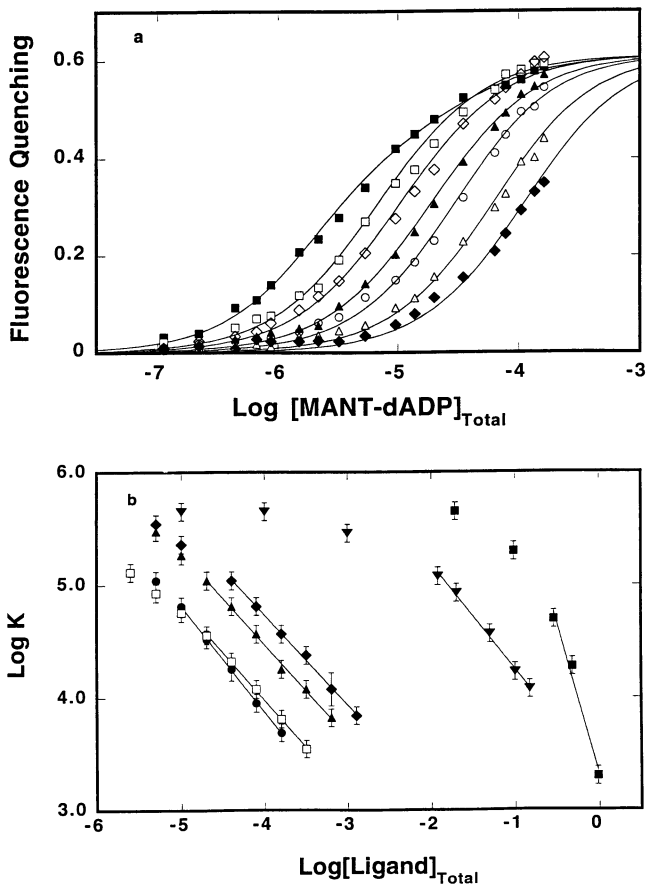

FIGURE 5 (*a*) Fluorescence titration of the DnaB hexamer with MANT-dADP, monitored by the quenching of the intrinsic protein fluorescence in buffer T2 (pH 8.1, 20 mM NaCl, 20°C) in the presence of different concentrations of GDP. Solid lines are computer fits obtained using a third-degree polynomial representing the exact relationship between the quenching of the protein fluorescence, Q, and the average degree of binding, $\Sigma\nu_i$ (Eq. 4 with $a = 14.1 \times 10^{-2}$, $b = -13.0186 \times 10^{-4}$, $c = 6.899 \times 10^{-4}$) and interaction parameters (hexagon model): 5×10^{-6} M GDP □, $K = 1.1 \times 10^5$ M^{-1}, $\sigma = 0.7$; 1×10^{-5} M GDP (◇), $K = 6.5 \times 10^4$ M^{-1}, $\sigma = 0.75$; 2×10^{-5} M GDP (▲), $K = 3.3 \times 10^4$ M^{-1}, $\sigma = 0.8$; 4×10^{-5} M GDP (●), $K = 1.8 \times 10^4$ M^{-1}, $\sigma = 0.9$; 8×10^{-5} M GDP (△), $K = 9 \times 10^3$ M^{-1}, $\sigma = 0.9$; 1.6×10^{-4} M GDP (◆), $K = 4.9 \times 10^3$ M^{-1}, $\sigma = 0.9$. (*b*) The dependence of the logarithm of the intrinsic binding constant of MANT-dADP upon the logarithm of the concentration of different nucleoside diphosphates, AMP, P_i, and NaBr. □, ADP; ●, GDP; ▲, UDP; ◆, CDP; ▼, P_i; ■, NaBr. The slopes of the linear part of the plots, $\partial\log K/\partial\log$[Ligand], at high competing ligand concentrations, are -0.95 ± 0.1 (GDP); -0.85 ± 0.1 (ADP); -0.85 ± 0.1 (UDP); -0.83 ± 0.1 (CDP); -0.95 ± 0.1 (P_i).

binding sites. As we pointed out, binding of the reference fluorescent analog to the hexamer, in the presence of a competing nonfluorescent nucleotide, is a three-state lattice binding system in which each binding site can exist in three different states, free, bound with a fluorescence analog, or bound with an unmodified nucleotide. Thus, the three-state lattice binding model provides an analytical description of the competition binding process (Eqs. 17–19). Because the values of K_1 and σ_1 can be independently determined from the titrations of the DnaB protein with the reference fluorescent nucleotide analog (MANT-dADP), there are only three unknown parameters in the model, K_2, σ_2, and σ_3. These parameters can be extracted by the nonlinear least-squares fit of the binding isotherm, using Eqs. 17–19. The analysis of the complex titration curves has also been greatly simplified by performing the fitting under conditions where the concentrations of both competing nonfluorescent nucleotide, $[L_2]_{Total}$, and reference fluorescent nucleotide, $[L_1]_{Total}$, are $\gg[DnaB]_{Total}$ at any titration point (Fig. 6). In such a situation, both $[L_2]_{Total}$ and $[L_1]_{Total}$ can be treated as practically free nucleotide concentrations, $[L_2]_{Free}$ and $[L_1]_{Free}$, and directly enter matrix Eqs. 17–19. Representative fluorescence titrations of the DnaB helicase with reference, fluorescent nucleotide analogs MANT-dADP, in the presence of different concentrations of ADP, are shown in Fig. 6. The solid lines are nonlinear least-squares fits of the isotherms to the three-state lattice binding model (Eqs. 16–19), which provide K_2 (ADP) $= (4.5 \pm 1.5) \times 10^5$ M^{-1}, $\sigma_2 = 0.15 \pm 0.05$, and $\sigma_3 = 0.25 \pm 0.07$. The intrinsic binding constant $K_1 = (4.2 \pm 1) \times 10^5$ M^{-1} and cooperativity parameter $\sigma_1 = 0.3 \pm 0.05$ for the binding of the reference ligand, MANT-dADP, have been independently determined (Fig. 3 *b*). The same analysis has been applied to all studied nucleoside diphosphates, AMP, and inorganic phosphate. The obtained intrinsic binding constants and cooperativity parameters are included in Table 2.

As in the case of modified fluorescent analogs, GDP binds with the highest intrinsic affinity, followed by ADP, UDP, and CDP. The intrinsic binding constants of unmodified nucleoside diphosphates are lower than the intrinsic affinities of corresponding MANT derivatives by a factor of ~2 in the case of purine nucleotides and a factor of ~3 in the case of pyrimidine nucleotides. All nucleoside diphosphates bind the DnaB hexamer with similar negative cooperativity ($\sigma_2 \approx 0.2 \pm 0.07$), which is close to the negative cooperativity of their corresponding MANT analogs (Table 1). These data suggest that the MANT group has a small additive effect on the intrinsic affinity and does not affect the interactions modulating the negative cooperativity. The intrinsic affinities of AMP and inorganic phosphate are dramatically lower (~2 orders of magnitude) than those of nucleoside diphosphates (Table 2). The affinity of AMP $K = (4.2 \pm 1) \times 10^2$ M^{-1} is very similar to the $K = (4.4 \pm 1) \times 10^2$ M^{-1} of inorganic phosphate. However, binding of both AMP and inorganic phosphate is characterized by negative cooperativity similar to the negative cooperativity observed in the binding of nucleoside diphosphates (Table 2).

DISCUSSION

The physiological role of the DnaB helicase is related to its interactions with ss- and dsDNA under the control of ATP binding and hydrolysis. Understanding the interactions between the helicase and nucleotide cofactors is a prerequisite for understanding the action of the enzyme. Our previous fluorescence spectroscopic studies have suggested contrast-

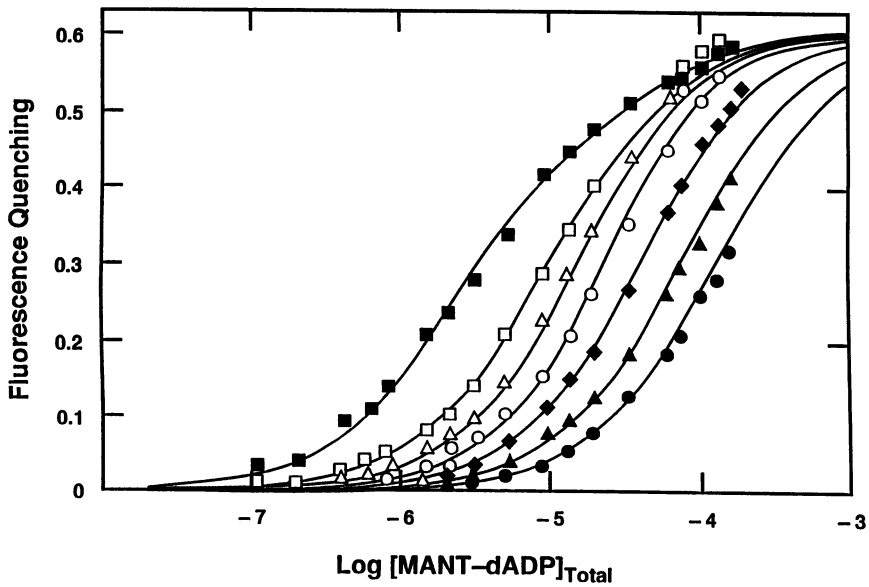

FIGURE 6 Fluorescence titration of the DnaB hexamer with MANT-dADP, monitored by the quenching of the intrinsic protein fluorescence in buffer T2 (pH 8.1, 20 mM NaCl, 20°C) in the presence of different concentrations of ADP. Solid lines are nonlinear least-squares fits obtained using a third-degree polynomial representing the exact relationship between the quenching of the protein fluorescence, Q, and the average degree of binding, $\Sigma\nu_i$ (Eq. 4 with $a = 14.1 \times 10^{-2}$, $b = -13.0186 \times 10^{-4}$, $c = 6.899 \times 10^{-4}$) and interaction parameters (three-state lattice model, Eqs. 16–19) for ADP binding, $K = 4.5 \times 10^5\ M^{-1}$, $\sigma_2 = 0.17$, $\sigma_3 = 0.23$. ADP concentrations are 0 M (■), 1×10^{-5} M (□), 2×10^{-5} M (△), 4.06×10^{-5} M (○), 8.12×10^{-5} M (◆), 1.6×10^{-4} M (▲), 3.2×10^{-4} M (●). The DnaB protein concentration is 5.4×10^{-7} M (hexamer).

ing differences between ribose and base binding regions of the nucleotide binding site of the DnaB helicase (Bujalowski and Klonowska, 1994b). The ribose binding region, which is most probably involved directly in inducing the allosteric transitions within the DnaB subunit, is practically immobilized with respect to the protein matrix and exists in two different conformations. On the other hand, the base of the bound nucleotide most probably senses a single environment and possesses relatively high mobility freedom, suggesting the lack of short-range specific interactions between the protein and the base. To assess the role of the base in nucleotide binding, we performed quantitative fluorescence titrations of the DnaB helicase with different MANT-modified nucleotide analogs, MANT-ADP, MANT-GDP, MANT-UDP, and MANT-CDP. The binding isotherms have been analyzed by using the statistical thermodynamic hexagon model to extract intrinsic affinities and cooperativities in the nucleotide binding (Eqs. 5–8; Bujalowski and Klonowska, 1993). Comparison of the intrinsic affinities shows that the DnaB helicase preferentially binds purine analogs with MANT-GDP having the highest intrinsic affinity. This affinity is ~1 order of magnitude higher than the affinity of the pyrimidine analog MANT-CDP (Table 1). Contrary to the intrinsic affinity, the negative cooperative interactions are similar for all studied analogs, indicating that the base does not affect negative cooperativity.

The analytical expressions for the partition function for the hexagon model, in which each binding site of the hexamer can exist in two states, free and bound, can be obtained by solving the eigenvalue problem of the 2×2 matrix of statistical weights (Eqs. 5–8). On the other hand, in a general case, each binding site can exist in an arbitrary number p of possible different states, and a $p \times p$ matrix of statistical weights is necessary to describe the system (Eq. 11). Obtaining analytical expressions for eigenvalues in such a general case would require a solution of the pth degree polynomial, which for $p > 2$ is a very difficult, if not impossible, task. Although the eigenvalues can be obtained through numerical calculations, a method of deriving analytical expressions for the partion function and degree of binding would greatly facilitate any fitting and computer simulation studies of the complex, multiple ligand binding systems. In this work we describe a method that can provide analytical formulation of the partition function and the degree of binding for the ligand-finite circular lattice system in which a binding site on the lattice can exist in an arbitrary number of states. This approach is based on the fact that, in the case of a circular lattice of n binding sites, the partition function can be defined in terms of the trace of matrix $\mathbf{M}_G^n$,

TABLE 2 Intrinsic binding constants, K_2, parameter characterizing cooperative interactions between bound nucleotide molecules of the same types, σ_2, and parameter describing the cooperative interactions between bound nucleotide molecules of different types, σ_3, for the competitive nucleotide binding to the DnaB hexamer in buffer T2 (pH 8.1, 20 mM NaCl, 20°C)

	ADP	GDP	UDP	CDP	AMP	P_i
K_2 (M^{-1})*	$(4.5 \pm 1) \times 10^5$	$(1.3 \pm 0.6) \times 10^6$	$(1.5 \pm 0.5) \times 10^5$	$(6 \pm 2) \times 10^4$	$(4.2 \pm 1) \times 10^2$	$(4.4 \pm 1) \times 10^2$
σ_2	0.15 ± 0.07	0.11 ± 0.07	0.23 ± 0.07	0.17 ± 0.07	0.15 ± 0.07	0.2 ± 0.07
σ_3	0.22 ± 0.05	0.25 ± 0.07	0.35 ± 0.5	0.27 ± 0.05	0.35 ± 0.1	0.35 ± 0.1

The parameters have been determined by nonlinear least-squares fits of Eqs. 18 and 19 to the experimental binding isotherms, using intrinsic binding constant $K_1 = 4.2 \times 10^5\ M^{-1}$ and $\sigma_1 = 0.3$ for the reference fluorescent ligand MANT-dADP.

* The errors associated with the determination of K and σ parameters are standard deviations obtained from four to six repeated titration experiments.

and each element of the trace can be defined in terms of the original matrix of statistical weights $\mathbf{M}_G$ (Eqs. 11–16).

Binding of unmodified nucleotides does not induce significant changes in the DnaB protein fluorescence (Bujalowski and Klonowska, 1993). Therefore, the estimation of the intrinsic affinities and the cooperativities in the interactions of these nucleotides with the helicase have been obtained using the competition titration approach with the reference fluorescent analog MANT-dADP. In the presence of two competing ligands, each nucleotide-binding site of the DnaB helicase can exist in three different states; thus, the matrix of statistical weights becomes a 3×3 matrix (Eq. 17). Although the model contains five interaction parameters, K_1, σ_1, K_2, σ_2, and σ_3, only three parameters cannot be independently determined, K_2, σ_2, and σ_3. However, the determination of these parameters can be accomplished by nonlinear least-squares fits of the analytical expressions to the experimental binding isotherms at different competing nucleotide concentrations (Eqs. 17–19). The obtained values of the binding parameters are included in Table 2. The DnaB helicase shows a significant preference for purine nucleotides, particularly GDP, which has an intrinsic affinity ~20-fold higher than the intrinsic affinity of CDP. Binding of each nucleotide is characterized by a negative cooperativity (σ_2 ~0.11 − 0.23), which is, as in the case of fluorescent analogs, similar for all studied nucleotides and is similar to the negative cooperativity in the binding of corresponding MANT derivatives (Tables 1 and 2). Comparison of the intrinsic affinities of MANT analogs and unmodified nucleotides indicates that the analogs have affinities that are higher by factors of 2–3. Thus, the MANT group does not affect negative cooperative interactions and provides only 0.4–0.6 kcal to the intrinsic free energy of binding of the modified nucleotides, indicating that MANT derivatives can serve as excellent analogs of the parent nucleotides in studies of the mechanism of the DnaB helicase (Bujalowski and Klonowska, 1993, 1994a,b; Bujalowski et al., 1994).

Both the preference for GTP and the capability of accepting and hydrolyzing all NTPs in its nucleotide-binding site may play an important role in the functioning of the DnaB protein. In the cell, the enzyme is delivered to the origin of replication site (oriC) in the complex with a specific transferase, DnaC protein (Kornberg and Baker, 1992). This complex is absolutely required to initiate replication, and its formation is specifically dependent upon ATP binding to the DnaC transferase (Wahle et al., 1989). No other nucleotide can replace ATP in this reaction. Current data indicate that the DnaC protein does not show any ATPase activity (Wahle et al., 1989). Thus the release of the ATP from the transferase could be efficiently achieved through transient, nucleotide exchange (e.g., with GTP) at the DnaC nucleotide binding site, thus triggering the subsequent release of the DnaB helicase from the complex with transferase and leaving the active DnaB helicase, which can use any NTP, to fuel the duplex DNA unwinding reaction at oriC.

Model-independent analysis of the fluorescence titrations of the DnaB helicase, with the reference fluorescent nucleotide analog (MANT-dADP), in the presence of an inorganic phosphate, shows $\partial \log K_{\text{MANT-dADP}}/\partial \log[\text{inorganic phosphate}] = -0.95 \pm 0.1$, indicating that only a single phosphate competes with the diphosphate analog for the binding of the helicase. The DnaB protein has a consensus nucleotide-binding site (Walker et al., 1982). Our data suggest that this phosphate-binding loop, which can accommodate three phosphate groups of bound ATP, has significant differences in affinities among phosphate-binding sites within the loop. We have previously indicated that only two phosphate groups of ATP contribute to the affinity for the nucleotide-binding site of the DnaB helicase (Bujalowski and Klonowska, 1993). The significant differences among phosphate-binding sites within the phosphate-binding loop can be a general property of this consensus sequence.

Analysis of the competition titration curves, using the three-state lattice model, allowed us to quantitatively estimate the intrinsic affinity of the inorganic phosphate and the cooperativity of the binding (Table 2). The intrinsic binding constant $K = (4.4 \pm 1) \times 10^2\ \text{M}^{-1}$ is ~2-3 orders of magnitude lower than the intrinsic binding constant of the nucleoside diphosphates. This significant, albeit lower, affinity does not seem to result from the lack of the base or sugar moiety, as AMP has the same intrinsic affinity as inorganic phosphate (Table 2), but rather the lack of an additional phosphate group seems to be a decisive factor, as indicated by the fact that the intrinsic affinity of pyrophosphate is already similar to the intrinsic affinities of NDPs (Jezewska and Bujalowski, manuscript in preparation). It should be noted that binding of inorganic phosphate is characterized by negative cooperativity similar to the negative cooperativity of NDPs, providing an additional indication that interactions in the phosphate-binding site, and not at the base-binding region, modulate negative cooperative interactions (Bujalowski and Klonowska, 1993).

Helicases belong to a class of motor proteins that transduce the free energy of NTP hydrolysis into mechanical work, i.e., translocation along DNA lattices. One of the fundamental features of the free energy transduction is that ATP must bind significantly stronger to the nucleotide-binding site than ADP and P_i, thus leading to a much lower free energy difference between ATP and the products of its hydrolysis. This decrease in the free energy difference between ATP and both ADP and P_i makes the hydrolysis of ATP readily reversible in the nucleotide-binding site and allows preservation of a large free energy change for energy transduction when the products are released (Jencks, 1980). Whether this mechanism is operational in the case of a helicase is still unknown. However, it is interesting that the affinities of the ATP analogs, MANT-AMP-PNP and ϵAMP-PNP, studied in this work are lower than the affinities of corresponding ADP analogs (Fig. 2, *a* and *b*). We have also previously determined that TNP-ATP binds with ~4-fold weaker intrinsic affinity than TNP-ADP (Bujalowski and Klonowska, 1993). Thus, the lower affinity of studied ATP analogs is independent of the location of the fluorescent modification and suggests that the affinity of ATP could be lower than that of ADP, in the case of the

DnaB helicase. This is different from another well-studied dimeric helicase, *E. coli* Rep protein, which has an affinity for ATP ~2 orders of magnitude higher than for ADP (Moore and Lohman, 1994). However, our preliminary data indicate that, in the presence of ssDNA, the affinity of ATP analogs for the nucleotide-binding site is significantly higher than the affinity of ADP analogs (Jezewska and Bujalowski, manuscript in preparation). These data suggest that the free energy transduction could be facilitated when the helicase forms a complex with ssDNA, in which the energetics of the hydrolysis reactions in the nucleotide-binding site is affected by the nucleic acid cofactor. At this time, no quantitative data are available on the energetics of ATP, ADP, and P_i binding for other hexameric helicases. However, it is very probable that forming a "holoenzyme" with ssDNA to acquire free energy-transducing capabilities is a general property of all hexameric replicative helicases.

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