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The *dna*B Protein of *Escherichia coli*: Mechanism of Nucleotide Binding, Hydrolysis, and Modulation by *dna*C Protein†

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ABSTRACT: The mechanism of nucleotide binding and hydrolysis by *dna*B protein and *dna*B·*dna*C protein complex has been studied by using fluorescent nucleotide analogues. Binding of trinitrophenyladenosine triphosphate (TNP-ATP) or the corresponding diphosphate (TNP-ADP) results in a blue shift of the emission maximum and a severalfold amplification of the fluorescence emission of the nucleotide analogues. Scatchard analysis of TNP-ATP binding indicates that TNP-ATP binds with a high affinity (K_d = 0.87 μM) and a 8.5-fold enhancement of fluorescence emission of the nucleotide. Only three molecules of TNP-ATP or TNP-ADP bind per hexamer of *dna*B protein in contrast to six molecules of ATP or ADP binding to a *dna*B hexamer. TNP-ATP and TNP-ADP are both competitive inhibitors of single-stranded (SS) DNA-dependent ATPase activity of *dna*B protein. TNP-AMP neither binds to *dna*B protein nor inhibits the ATPase activity. Formation of *dna*B·*dna*C complex by *dna*C protein results in diminution of the TNP-ATP fluorescence enhancement and a concomitant decrease in the SS DNA-dependent ATPase activity. Kinetic analysis of the ATPase activity of *dna*B·*dna*C complex indicates that the decrease in the ATPase activity on complex formation is due to a reduction of the maximal velocity (V_{max}). The *dna*B protein hydrolyzes both TNP-ATP and dATP, however, with an extremely slow rate in the presence of single-stranded M13 DNA. The 2′-OH group of the nucleotide most likely plays an important role in the hydrolysis reaction but not in the nucleotide binding.

The *dna*B protein is a major component of the chromosomal DNA replication in *Escherichia coli* (Kornberg, 1980). The *dna*B protein, a hexamer of 52000-dalton identical subunits (Arai et al., 1981a; Reha-Krantz & Hurwitz, 1978a,b; Nakayama et al., 1984), is required in the initiation (Baker et al., 1986), priming, and elongation stages of DNA replication (Arai & Kornberg, 1981b; Arai et al., 1981b; Zyskind & Smith, 1977; Wechsler & Gross, 1971; McMacken & Kornberg, 1978). In vitro DNA replication studies with DNA from

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bacteriophages and plasmids demonstrated the multiple activities of this protein: (i) rNTP and dNTP binding; (ii) DNA-dependent rNTPase (Reha-Krantz & Hurwitz, 1978b; Arai & Kornberg, 1981a); (iii) single- and double-stranded DNA binding (Arai & Kornberg, 1981); (iv) interaction with *dna*C protein (Wickner & Hurwitz, 1975; Kobori & Kornberg, 1982), *dna*G protein (primase) (Arai & Kornberg, 1981b), and λ-encoded P protein (Wickner, 1978). The *dna*B protein is thus capable of interacting with various ribo- and deoxynucleotides, single- and double-stranded DNA. It has been discovered recently that *dna*B protein functions also as a helicase, unwinding DNA in the replication fork (Lebowitz & McMacken, 1986; Baker et al., 1986).

The *dna*C is an essential gene in *E. coli*. The *dna*C gene product, a monomeric 29 000-dalton polypeptide, is required for chromosomal DNA replication in *E. coli* in vitro and in vivo (Kobori & Kornberg, 1982a,b; Kornberg, 1980). The essential roles of *dna*C protein remain unknown. However, *dna*C protein forms a tight, isolatable complex with *dna*B protein in the presence of ATP or dATP, but not in the presence of nonhydrolyzable ATP analogues such as ADP, 5′-adenylyl imidodiphosphate (AMPPNP),[1] AMP, etc. (Kobori & Kornberg, 1982b). The *dna*B·*dna*C complex (BC complex), with a 1:1 molar ratio of *dna*B and *dna*C proteins, is likely functional in the chromosomal DNA replication. However, the mechanism of action of the BC complex is still unclear.

In this paper, the dynamics of the interaction of the *dna*B protein with nucleotides have been investigated by using a fluorescent analogue of ATP, the 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidine)adenosine 5′-triphosphate (TNP-ATP). The TNP-nucleotides are environmentally sensitive probes for the nucleotide binding sites in enzymes (Hiratsuka & Uchida, 1973; Moczydlowski & Forbes, 1981a,b; Grubmeyer & Penefsky, 1981a,b). The binding of TNP-ATP to a hydrophobic site results in a severalfold enhancement of its fluorescence emission. This property of TNP-nucleotides makes them attractive probes for the nucleotide binding sites of such enzymes. The effectiveness of using these fluorescent nucleotide analogues as probes of protein–nucleotide interaction has been demonstrated with myosin ATPase (Hiratsuka & Uchida, 1973), the (Na^+,K^+)-ATPase (Moczydlowski & Fortes, 1981a,b), the F_1-ATPase (Grubmeyer & Penefsky, 1981a,b), aspartokinase I (Broglie & Takahashi, 1983), and sarcoplasmic reticulum Ca^{2+}-ATPase (Dupont et al., 1982; Bishop et al., 1985).

We have used various TNP-nucleotides to investigate the interaction of *dna*B protein with nucleotides, an essential step in its mechanism of action. We have evaluated the role of *dna*C protein in nucleotide binding and hydrolysis by *dna*B protein in the *dna*B·*dna*C complex.

Materials and Methods

Chromatographic Materials. PEI–cellulose plates were purchased from J. T. Baker Chemical Co. Lithium chloride and formic acid were purchased from Sigma and J. T. Baker, respectively. DEAE-cellulose and phosphocellulose were from Whatman, and Sephacryl S-300 was from Pharmacia.

[1] Abbreviations: TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)adenosine 5′-triphosphate; TNP-ADP, 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)adenosine 5′-diphosphate; TNP-AMP, 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)adenosine 5′-monophosphate; PEI, poly(ethylenimine); DTT, dithiothreitol; BSA, bovine serum albumin; SS DNA, single-stranded DNA; BC complex, the *dna*B·*dna*C protein complex of equimolar ratio; AMPPNP, 5′-adenylyl imidodiphosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

Proteins, Enzymes, and DNA. *dna*B protein (1×10^6 units/mg) was purified from YS1recA(pKA1) as described (Arai et al., 1981a); *dna*C protein was obtained as a gift from Drs. J. Flynn and A. Kornberg of Stanford University (Kobori & Kornberg, 1983a). M13 phage DNA was prepared as described (Ray, 1969).

Nucleotides. ATP and dATP were purchased from P-L Biochemicals. [^{3}H]ATP and [^{32}P]dATP were obtained from New England Nuclear. TNP-ATP was purchased from Molecular Probes, Eugene, OR. TNP-ATP, TNP-ADP, and TNP-AMP were kindly supplied by Dr. R. Nakamoto of this department. All TNP-nucleotides were purified by the method of Hiratsuka (1982) and were examined for purity by thin-layer chromatography or by high-performance liquid chromatography. [^{3}H]TNP-ATP was prepared following the method of Hiratsuka (1982). Concentrations of TNP-nucleotides were determined by using the molar extinction coefficient $E_{408} = 26\,400\ M^{-1}\ cm^{-1}$ and $E_{470} = 18\,500\ M^{-1}\ cm^{-1}$ in 0.1 M Tris-HCl (pH 8.0) (Hiratsuka, 1982).

Buffers. Buffer A is 50 mM Tris-HCl (pH 7.6), 10% (w/v) sucrose, and 5 mM $MgCl_2$. In experiments involving *dna*C protein, buffer A also contained 5 mM DTT. Buffer B contained 100 mM Tris-HCl (pH 7.5), 20% (w/v) sucrose, 10 mM DTT, and 40 μg/mL BSA.

Fluorescence Measurements. Fluorescence measurements were performed on an Aminco-Bowman spectrofluorometer for all steady-state fluorescence experiments, where the wavelengths of excitation and emission remained constant. For all TNP-nucleotide fluorescence experiments, excitation was set at 410 nm and emission at 545 nm. All titrations were carried out in a 0.5 cm × 1 cm glass cuvette to minimize inner filter effects, and a linear fluorescence response was observed up to 10 μM TNP-ATP. The fluorescence emission spectra and the titration of TNP-ATP with *dna*B protein were performed by using a Varian SK digital spectrofluorometer; the excitation wavelength was 410 nm. The *dna*B titrations were carried out by adding 0.5-μL aliquots of 10 mg/mL *dna*B protein to 0.5 μM TNP-ATP or TNP-ADP. The concentration of nucleotide was maintained by adding 0.5 μL of 1 μM nucleotide following the addition of protein.

ATPase Assays. This assay measures production of [^{3}H]ADP from [^{3}H]ATP on ATP hydrolysis. In a standard assay, 20 μL of the reaction mixture was prepared at 0 °C and contained 10 μL of buffer B, 10 mM $MgCl_2$, 500 pmol (as nucleotide) of single-stranded M13 phage DNA, 160 ng of *dna*B (or as indicated), and 100 μM [^{3}H]ATP (2000 cpm/pmol) (or as indicated). The reactions were incubated at 30 °C. The reactions were terminated by adding 2 μL of 200 mM EDTA followed by chilling in ice. Two-microliter aliquots were applied to PEI–cellulose strips which were prespotted with ADP-ATP marker. The strips were developed with 1 M formic acid/0.5 M LiCl and dried, and the ATP and ADP spots were located by UV fluorescence. The portions containing ATP and ADP were excised and counted in a toluene-based scintillator with a Packard Tri-Carb liquid scintillation spectrometer. The rate of hydrolysis is linear up to 10 min in such assays.

In the inhibition studies, the appropriate inhibitors were added to the reaction mixture at 0 °C, prior to incubation, in the indicated amount. In studies involving *dna*C, the complex was preformed by incubation of *dna*B and *dna*C proteins at 0.64 mg/mL *dna*B and the indicated amounts of *dna*C protein in the presence of 10 μM ATP at 30 °C for 5 min. The ATPase assay was then carried out by adding 0.25 μL of the enzyme mix to 19.7 μL of the reaction mixture. The remainder

of the assay was carried out in the standard manner as described above.

Results

Interaction of TNP-Nucleotides with dnaB Protein. TNP-nucleotides function as reporters of the nucleotide binding site environment in an enzyme (Hiratsuka & Uchida, 1973; Moczydlowski & Fortes, 1981a,b). The absorption at 410 nm by the TNP-nucleotide remains more or less unaffected by the environment of the TNP moiety (Moczydlowski & Fortes, 1981a). However, the emission spectrum of a TNP-nucleotide is extremely sensitive to the environment (Moczydlowski & Fortes, 1981a). A decrease in polarity of the solvent increases the relative quantum yield drastically with a concomitant blue shift of the emission maximum. The viscosity of the solvent also increases the relative quantum yield but tends to decrease the blue shift of the emission maximum.

The observed emission maximum of TNP-ATP alone in buffer A is 559 nm which is comparable to that observed in H_2O (Moczydlowski & Fortes, 1981a). The presence of sucrose does not alter the emission maximum (data not shown); however, it causes approximately 3-fold enhancement of the fluorescence. The addition of *dna*B protein results in a severalfold enhancement of the fluorescence and a blue shift of the emission maximum to 547 nm. A comparison of the emission spectrum of TNP-ATP in *dna*B·TNP-ATP complex with those of TNP-ATP in solvents of different polarity (Moczydlowski & Fortes, 1981a) indicates that this emission spectrum is comparable to that observed in glycerol. The fluorescence enhancement and the emission maximum of 547 nm suggest that the TNP-ATP binding site in *dna*B is more hydrophobic than H_2O, perhaps similar to that of methanol or glycerol.

Figure 1A shows a typical TNP-ATP fluorescence titration in the presence and absence of the enzyme. The titration shows a high-affinity fluorescence enhancement, and this enhancement apparently saturates since the slopes of the plots of the fluorescence vs. TNP-ATP concentration in the presence and absence of *dna*B protein become identical at high TNP-ATP concentration. Addition of high levels of ATP results in a reversal of the fluorescence enhancement (data not shown). This evidence suggests binding of TNP-ATP to the nucleotide binding site of the *dna*B protein.

The fluorescence enhancement of TNP-ATP, TNP-ADP, and TNP-AMP on binding to the *dna*B protein is presented in Figure 1B. Among these three nucleotides, the fluorescent enhancement is maximum with TNP-ATP. In the case of TNP-ADP, the enhancement saturates; however, the enhancement is only 50% of that observed with TNP-ATP. TNP-AMP does not show any enhancement at lower concentrations of the nucleotide (less than equal to 10 μM). The half-maximal fluorescence enhancement is observed at 1.0 μM for TNP-ATP and at 0.75 μM for TNP-ADP. The observed difference in the fluorescence enhancement of TNP-ATP and TNP-ADP could possibly be due to a difference in the modes of binding of the two nucleotides by the enzyme. However, it is apparent from these data that the three phosphate moieties in the triphosphate part of TNP-ATP play an important role in the binding of the nucleotide to the enzyme.

Number of TNP-ATP Binding Sites in the dnaB Protein. The fluorescence titration of *dna*B protein by TNP-ATP was carried out as described in the previous section. The titration data have been analyzed to determine the K_d of the E·TNP-ATP complex and the number of TNP-ATP binding sites per *dna*B hexamer according to the technique described by Mas and Coleman (1985).

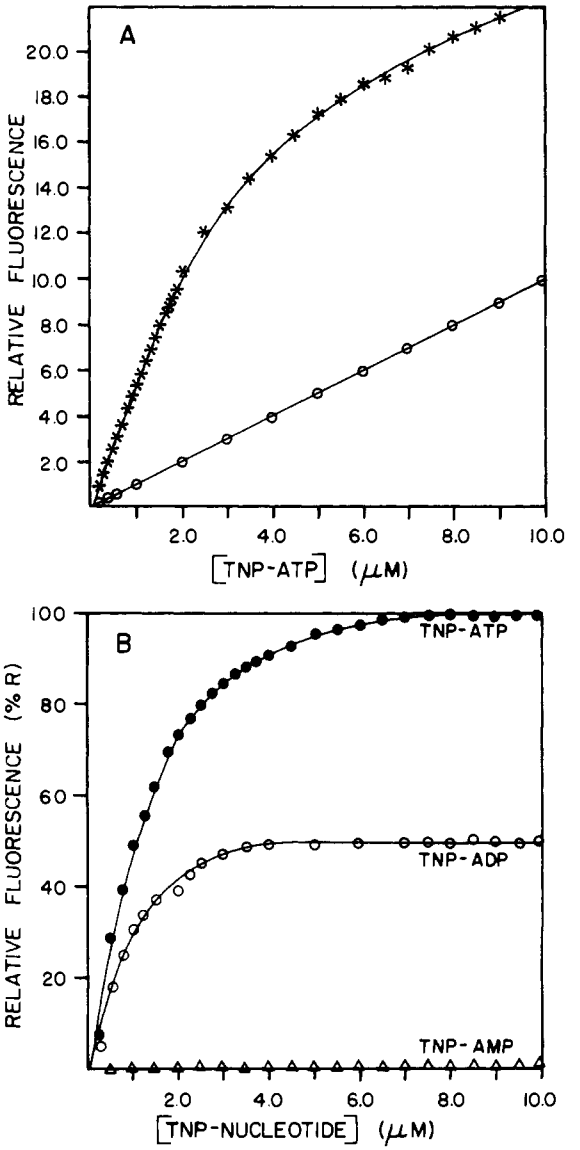

FIGURE 1: (A) Fluorescence titration of TNP-ATP in the presence (asterisks) and absence of *dna*B protein (4.8 μM) in buffer A; (B) fluorescence titration of *dna*B protein (4.8 μM) with TNP-ATP (●), TNP-ADP (○), and TNP-AMP (△) in buffer A. All experiments were carried out with 410 nm as the excitation wavelength and 545 nm as the emission wavelength.

The observed fluorescence (F) is the sum of the fluorescence of the free and bound ligand:

$$F = F_{free}[L]_{free} + F_{bound}[L]_{bound} \quad (1)$$

The fluorescence enhancement factor (Q) and the dissociation constant (K_d) are expressed as

$$Q = F_{bound}/F_{free} - 1 \quad (2)$$

$$K_d = [E]_{free}[L]_{free}/[L]_{bound} \quad (3)$$

If F and F_0 are the total observed fluorescence of the ligand in the presence and absence of the enzyme, E, then eq 1–3 could be used to derive the following relationship between F, F_0, K_d, and Q:

$$1/(F/F_0 - 1) = (K_d/Q)(1/[E]_{free}) + 1/Q \quad (4)$$

At sufficiently high enzyme concentration relative to the ligand concentration and the dissociation constant, one may approximate that the concentration of the free enzyme will approach that of the total enzyme. Therefore, a plot of $1/(F/F_0 - 1)$ vs. $1/[E]_{total}$ will provide the value of Q.

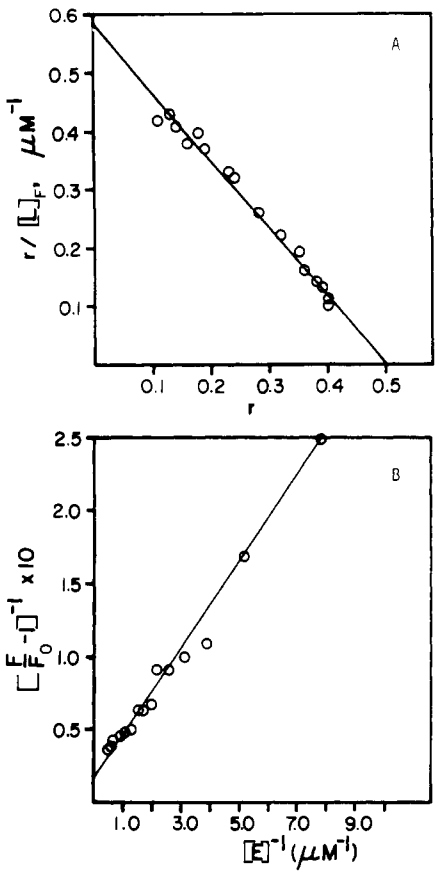

FIGURE 2: Fluorescence determination of physical parameters of the *dna*B–TNP-ATP interaction. (A) Scatchard analysis of the fluorescence titration of *dna*B by TNP-ATP (as shown in Figure 1A) by using the Scatchard equation, $r/[L]_{free} = n/K_d - r/K_d$ where r is the ratio of bound ligand to the total protein concentration and n is the number of binding sites per monomer. The plot is a least-squares fit with K_d = 0.87 μM and n = 0.5. (B) Determination of the fluorescence enhancement factor Q by titration of 0.5 μM TNP-ATP with *dna*B protein at enzyme concentrations ranging from 0.064 to 1.92 μM (monomer concentration). The value of Q determined from this plot is 7.5 (see text for details of calculation). The line is drawn on the basis of least-squares fit of all the points.

Figure 2B shows a plot for the titration of 0.5 μM TNP-ATP with *dna*B protein at enzyme concentrations ranging from 0.064 to 1.92 μM. The enhancement factor for TNP-ATP is 7.5 (eq 2), indicating approximately 8.5-fold enhancement of the fluorescence of TNP-ATP by *dna*B protein. The value of K_d obtained from this plot is invariably erroneous due to an error arising from the above approximation. The concentrations of the free and bound ligands at any given point in a titration as shown in Figure 1A could be evaluated from the equation:

$$[L]_{bound} = [L]_{total}(F/F_0 - 1)/(Q - 1) \quad (5)$$

The dissociation constant of the *dna*B·TNP-ATP complex was then determined from the Scatchard plot shown in Figure 2A. The K_d is 0.87 μM which is comparable to that determined from a simple fluorescence titration shown in Figure 1B.

The value of n, the number of binding sites per enzyme monomer, as determined from the Scatchard plot in Figure 2A is 0.5. As the native *dna*B protein is a hexamer, therefore, only three molecules of TNP-ATP can bind per hexamer. A nitrocellulose filter binding assay with radiolabeled [^{3}H]-TNP-ATP gave a K_d of 1.2 × 10^{-6} M, and Scatchard analysis indicated approximately three TNP-ATP binding sites per hexamer of *dna*B. A similar fluorescence analysis with TNP-ADP showed the enhancement factor Q is 4.0, K_d is 2.8 μM, and n is 0.6 per monomer (data now shown). Arai and Kornberg (1981c) demonstrated by equilibrium gel filtration that the number of binding sites for ATP, ADP, and ATPγS is six per hexamer. The TNP derivatives are much bulkier than the nucleoside triphosphates. As a result, although all six sites in a hexamer can accomodate ATP or ADP, only three sites can accomodate TNP-ATP in the concentration range that we have studied. Binding to other sites at a different concentration range cannot be ruled out.

Table I: Kinetic Parameters of Nucleotide Hydrolysis by *dna*B Protein and *dna*B·*dna*C Complex[a]

	*dna*B			*dna*B·*dna*C complex[b]	
	K_i (μM)	K_m (μM)	V_{max} (pmol min^{-1} mg^{-1})	K_m	V_{max}
ATP		100	1.2 × 10^7	70	3.9 × 10^6
dATP		60	1.1 × 10^5		
TNP-ATP	60		1.8 × 10^5		
TNP-ADP	90				
TNP-AMP	>1000				

[a] The kinetic parameters (K_i, K_m, and V_{max}) were evaluated as described in the text using 8 μg/mL *dna*B protein. All reactions were carried out at 30 °C. [b] The *dna*B·*dna*C complex was performed in the presence of ATP and was isolated by gel filtration in Bio-Gel A 0.5m in buffer A (Kobori & Kornberg, 1982b).

TNP-Nucleotides Are Inhibitors of SS DNA-Dependent ATPase Activity of dnaB Protein. TNP-ATP is hydrolyzed by myosin ATPase (Hiratsuka & Uchida, 1973) and by bovine heart mitochondrial ATPase (Grubmeyer & Penefsky, 1981a,b); however, it is not a substrate for eel electroplax (Na,K)-ATPase (Moczydlowski & Fortes, 1981a,b). In the case of sarcoplasmic reticulum Ca^{2+}-ATPase, it is hydrolyzed in a Ca^{2+}-independent nonspecific fashion with an extremely slow rate. TNP-ATP is hydrolyzed by the *dna*B protein with an extremely slow rate (Table I). Under standard reaction conditions, TNP-ATP was hydrolyzed in an SS DNA-dependent manner with a rate of 1.8 × 10^5 pmol min^{-1} (mg of *dna*B protein)$^{-1}$, as measured by the release of inorganic phosphate (Lin & Morales, 1977). This rate is approximately 1.5% of that observed with ATP itself and closely comparable to that observed with dATP. Deoxy-ATP lacks the 2′-OH group of the ribose, and in TNP-ATP, both 2′- and 3′-OH groups are blocked; therefore, the ribose 2′- and 3′-OH groups may play important roles in the mechanism of ATP hydrolysis of *dna*B protein.

Although the rate of hydrolysis of TNP-ATP is substantially slower than that of ATP, our result confirms that TNP-ATP is binding to the ATPase site of the *dna*B protein.

TNP-ATP and TNP-ADP are both competitive inhibitors of ATP hydrolysis. Figure 3 shows double-reciprocal plots of *dna*B ATPase activity in the presence of TNP-ATP. TNP-ATP inhibits the SS DNA-dependent ATPase activity of *dna*B protein competitively by altering the apparent K_m of the reaction. A plot of the apparent K_m ($K_{m,app}$) vs. inhibitor concentration indicates that K_i for TNP-ATP is 60 μM. A similar competitive inhibition is observed with TNP-ADP. The K_i for TNP-ADP is 90 μM (Table I), and it has no apparent effect on the V_{max} of the ATPase activity. However, in the case of TNP-AMP, no measurable inhibition is observed.

Modulation of Nucleotide Binding and Hydrolysis by dnaC Protein. The *dna*C gene product, a 29 000-dalton monomer,

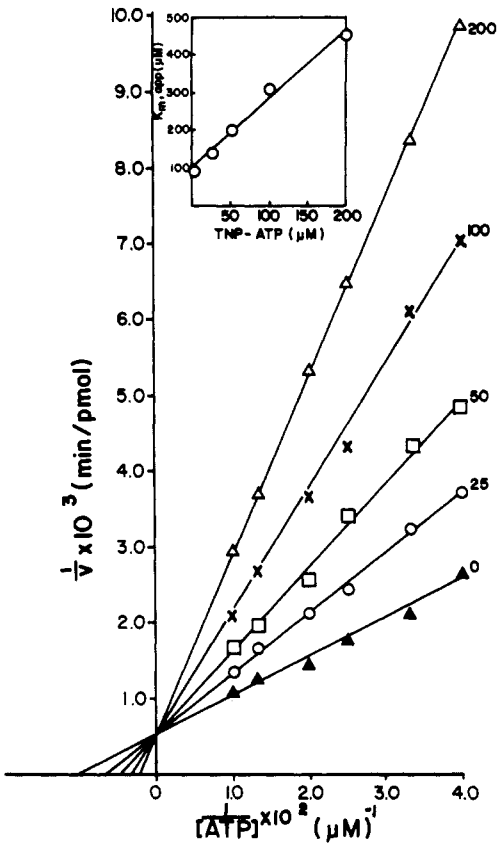

FIGURE 3: Lineweaver–Burk plot of TNP-ATP inhibition of SS DNA-dependent ATPase activity of the *dna*B protein. The assays were carried out as described except that TNP-ATP was added in the amount (0–200 μM) indicated with each plot. All calculations were based on least-squares analyses of the data. The presented data were derived by averaging three independent kinetic analyses. The insert is a plot of the apparent K_m vs. [TNP-ATP]. The value of K_m for ATP is 100 μM, and K_i for TNP-ATP is 60 μM.

forms a tight macromolecular complex with *dna*B protein in the presence of ATP and Mg^{2+} (Kobori & Kornberg, 1982b). The effect of such complex formation on the ATPase activity remains unclear. Kobori and Kornberg (1982a) did not find any inhibition, although Arai and Kornberg (1981a) reported inhibition of ATPase activity by *dna*C protein that depended on the nature of the DNA effector.

Our results indicate that the *dna*B·*dna*C complex formation resulted in the inhibition of the SS DNA-dependent ATPase activity of the *dna*B protein. A titration of the *dna*C protein inhibition of the SS DNA-dependent ATPase activity is shown in Figure 4. At saturating levels of *dna*C protein, the maximal inhibition is 63%. A double-reciprocal plot of the SS DNA-dependent ATPase activity of the *dna*B·*dna*C complex is shown in Figure 4. With preformed BC complex (Kobori & Kornberg, 1982b), the K_m for ATP is 70 μM, and the maximal velocity is 3.9×10^6 pmol min^{-1} mg^{-1} at 30 °C. The V_{max} is reduced approximately 67% on complex formation with *dna*C protein, compared to that of *dna*B protein alone. The binding of ATP, as evidenced from a lower K_m, is tighter than with *dna*B alone. Our recent photoaffinity labeling studies indicate that *dna*C protein directly participates in the ATP binding to *dna*B protein in the BC complex.

The binding of *dna*C protein to *dna*B·TNP-ATP complex has a profound effect on the fluorescence emission of TNP-ATP in the bound state. Fluorescence titration of *dna*B·TNP-ATP complex by *dna*C protein is shown in Figure 5. The addition of one monomer of *dna*C protein to a hexamer of *dna*B protein results in a 32% quenching of the TNP-ATP

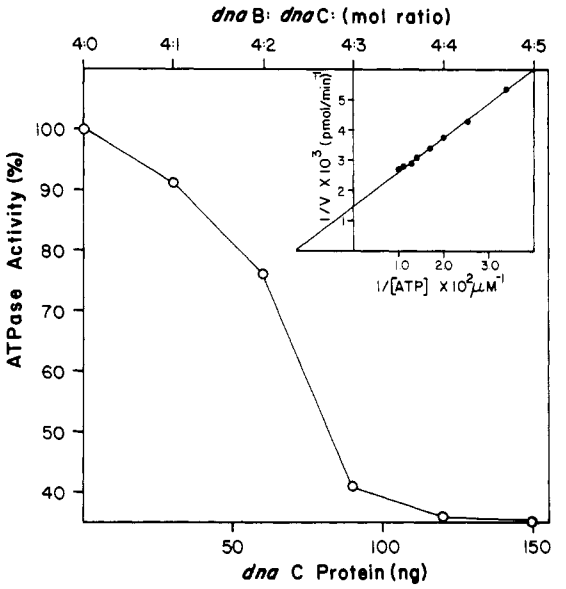

FIGURE 4: Inhibition of SS DNA-dependent ATPase activity of *dna*B protein by *dna*C protein. Assays were carried out as described under Materials and Methods. *dna*B and *dna*C were preincubated with 10 μM ATP (to preform the BC complex). The assay was carried out by adding 0.25 μL of BC complex to 19.75 μL of the reaction mixture at 30 °C for 5 min. Insert: Analysis of SS DNA-dependent ATPase activity of the *dna*B·*dna*C protein complex. The assays were carried out with 0.26 μg of complex (isolated by gel filtration chromatography in Sephacryl S-300) in a standard assay as described under Materials and Methods, except that *dna*B was omitted from the standard assay. The K_m for ATP, as determined from this plot, is 70 μM, and the V_{max} is 3.9×10^6 pmol min^{-1} mg^{-1} at 30 °C.

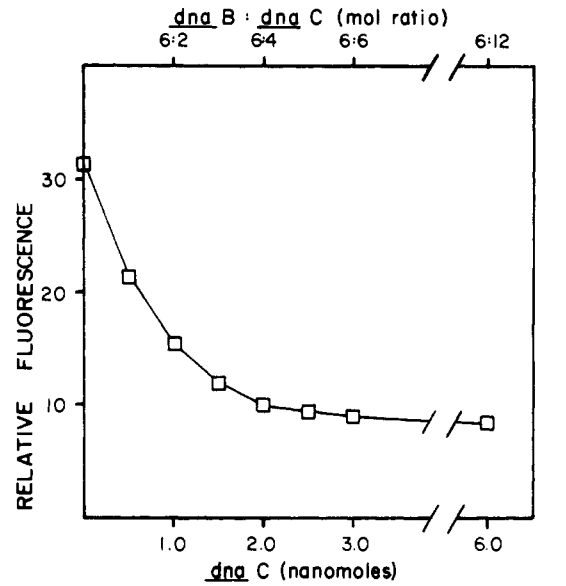

FIGURE 5: Fluorescence enhancement titration of *dna*B protein (2 μM) and TNP-ATP (3 μM) with *dna*C protein in buffer A. No fluorescence changes were observed in this titration in the absence of *dna*B protein.

fluorescence. The quenching of fluorescence is significant also during the addition of a second or third monomer of *dna*C to *dna*B protein. Further addition of *dna*C to the complex results in minor and insignificant quenching of the fluorescence. However, even at complete saturation (at greater than the equimolar ratio of *dna*C to *dna*B), a residual fluorescence, 30% of that observed in the complete absence of *dna*C, is observed. In addition, photo-cross-linking of ATP to *dna*B decreases approximately 3-fold on addition of saturating levels of *dna*C, a phenomenon that clearly supports the decrease of ATP or TNP-ATP binding to *dna*B.[2] This residual fluorescence is

completely insensitive to the addition of large excess of ATP. The *dna*C protein has no quenching effect on the fluorescence emission of TNP-ATP alone (data not shown). Addition of preformed *dna*B·*dna*C complex (formed in the presence of ATP and isolated by gel filtration) to TNP-ATP results in no significant changes of TNP-ATP fluorescence intensity. Therefore, the number of TNP-ATP binding sites in the BC complex could not be determined. The complex formation, most likely, results in the alteration of the ATP binding site in the *dna*B protein that allows ATP to bind, however not its bulkier analogue TNP-ATP. However, the residual TNP-ATP fluorescence observed during titration of *dna*B·TNP-ATP complex with *dna*C protein indicates that most likely one of the three TNP-ATP molecules remains bound per hexamer and this molecule cannot be exchanged with ATP alone. Our results indicate that *dna*C modulates the binding of nucleotide to *dna*B protein significantly, and the nucleotide binding characteristics of the BC complex are likely different from *dna*B protein alone. It is interesting to note that the λ P protein forms a complex with *dna*B protein, similar to that observed with *dna*C protein, and this complex formation abolishes the ATPase activity of the *dna*B protein (R. McMacken, personal communication).

SS DNA-Dependent dATPase Activity of dnaB Protein. The *dna*B protein hydrolyzes ATP and other ribonucleotides at varying rates (Arai & Kornberg, 1981a). However, it is also capable of hydrolyzing deoxy-ATP with SS DNA as a cofactor. The rate of dATP hydrolysis by *dna*B protein is much less than that observed with ATP alone. A Lineweaver–Burk plot of dATP hydrolysis indicates that the K_m of dATP is 60 μM and the V_{max} is 1.1×10^5 pmol min^{-1} mg^{-1} at 30 °C (Table I). Consequently, the 2′-OH of ATP has an insignificant effect on the binding of ATP as evidenced from the values of K_m of ATP and dATP. However, the 2′-OH of ATP may have specific interaction with the protein residues in the active site of *dna*B in order to alter the rate of nucleotide hydrolysis so significantly.

Discussion

The *dna*B protein is a multifunctional DNA replication enzyme (Kornberg, 1980). Among its many unique properties, it has the ability to hydrolyze ribonucleotides in a DNA-dependent manner. We have probed its interaction with ATP and the ribonucleotides in general by studying its interaction with TNP-ATP, a fluorescent analogue of ATP.

TNP-ATP binding to *dna*B protein results in (i) a blue shift of the emission maximum of the TNP moiety from 559 to 547 nm and (ii) an 8.5-fold enhancement of fluorescence. The fluorescence characteristics of TNP-nucleotides are extremely sensitive to their environment. It has been shown by Moczydlowski and Fortes (1981a) that hydrophobicity and viscosity of solvent alter the fluorescence spectrum of TNP-ATP dramatically. A comparison of the fluorescence spectrum of the *dna*B·TNP-ATP complex with the solvent perturbation spectra of TNP-ATP (Moczydlowski & Fortes, 1981a) indicates that the binding site of TNP-ATP is significantly hydrophobic, comparable to that observed in glycerol. The change in emission maximum in the *dna*B·TNP-ADP complex is similar to that observed with TNP-ATP; however, the enhancement is significantly reduced (Figure 1), indicating a difference in the modes of binding of the two nucleotides to *dna*B protein.

An interesting observation is the strong affinity of *dna*B protein toward TNP-ATP and TNP-ADP whereas it appears to lack any affinity toward TNP-AMP as determined by fluorescence studies (Figure 1) and by inhibition of ATPase activity of *dna*B protein by TNP-nucleotides (Table I). Thus, the three phosphate groups may play important roles in the recognition and binding of nucleotides by *dna*B protein.

A Scatchard analysis of TNP-nucleotide binding to *dna*B protein (Figure 2) indicates that only three molecules of TNP-ATP or TNP-ADP bind per *dna*B hexamer. However, Arai and Kornberg (1981c) reported six ATP or ADP binding sites per hexamer. A likely possibility is the presence of two classes of ATP binding sites, both of which can accommodate ATP; however, only one class can accommodate bulkier analogues such as the TNP-nucleotides. *dna*B protein binds single- and double-stranded DNA and functions as a helicase (Lebowitz & McMacken, 1986). The DNA binding site(s) of *dna*B protein remain(s) unknown. However, it is perhaps possible that one or more of the nucleotide triphosphate binding sites may also play a role in the polynucleotide binding in the replication fork. Consequently, the two classes of binding sites may have special roles in the replication fork. Further studies are required in order to address this important question.

TNP-ATP binds *dna*B protein with a dissociation constant of 0.87 μM (Figure 2), comparable to that observed with other nucleotides (Arai & Kornberg, 1981a). However, its rate of hydrolysis is extremely low, approximately 1.5% of that observed with ATP itself. *dna*B protein also hydrolyzes dATP in a DNA-dependent manner with a diminished rate, comparable to that observed with TNP-ATP (Table I).[3] The K_m of dATP hydrolysis (60 μM) is slightly lower than that observed with ATP (100 μM). Therefore, the binding of ATP and dATP to the *dna*B protein is similar, and the difference in the nucleotide hydrolysis is a result of a significant change in the V_{max}. TNP-ATP is blocked at the 2′- and 3′-positions due to chemical modification, and dATP lacks the 2′-OH group. Consequently, it is possible that the 2′-OH group of ribose interacts with the enzyme in its active site and facilitates the hydrolysis reaction.

The *dna*C gene product, a monomer of 29 000 daltons, forms a tight complex with *dna*B protein (Kobori & Kornberg, 1982b). The *dna*B·*dna*C complex then functions in the replication fork. Binding of *dna*C results in an alteration of the rate of nucleotide hydrolysis. Our kinetic analysis indicates a 67% reduction of the rate (Figure 4). This diminution of rate is solely due to the lowering of V_{max} by *dna*C protein (Figure 4) in the BC complex. The interaction of *dna*C with the *dna*B·TNP-ATP complex results in a reduction of fluorescence intensity (Figure 5), and at saturating levels of *dna*C, a 30% residual fluorescence was observed. The effects of *dna*C interaction with *dna*B protein are comparable in both the ATP hydrolysis and the enhancement of TNP-ATP fluorescence. Thus *dna*C protein likely functions as a modulator of nucleotide binding and hydrolysis by *dna*B protein. Our recent studies using a photoaffinity labeling procedure (Biswas & Kornberg, 1984) indicate that *dna*C participates directly in the nucleotide binding by *dna*B protein and modulates the nucleotide binding and hydrolysis.[2]

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[2] S. B. Biswas and E. Biswas, unpublished results.

[3] McMacken and co-workers found a similar dATPase activity of *dna*B protein (R. McMacken, personal communication).

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Registry No. ATP, 56-65-5; dATP, 1927-31-7; TNP-ATP, 97902-34-6; TNP-ADP, 97902-37-9; ATPase, 9000-83-3.

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