Characterization of the Nucleotide Binding Properties of SV40 T Antigen Using Fluorescent 3′(2′)-*O*-(2,4,6-Trinitrophenyl)adenine Nucleotide Analogues[†]

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ABSTRACT: ATP binding to the large tumor (T) antigen encoded by the simian virus 40 (SV40) genome plays an essential role in the replication of viral DNA [Fanning, E., and Knippers, R. (1992) *Annu. Rev. Biochem. 61*, 55−85]. To better explore the functions of T antigen during the replication process, we have studied the interactions of T antigen with fluorescent 3′(2′)-*O*-(2,4,6-trinitrophenyl) (TNP) adenine nucleotide analogues. Binding of TNP-ATP and TNP-ADP was accompanied by an 8-fold fluorescence enhancement and a concomitant blue shift (11 nm) of the maximal emission wavelength; the intrinsic protein tryptophan fluorescence was quenched maximally by 50%. Both signals were utilized to characterize the nucleotide binding activity of T antigen. TNP-ATP and TNP-ADP bound to the ATP binding site with dissociation constants of 0.35 μM and 2.6 μM. TNP substitution enhanced the affinity of ADP for T antigen by approximately 11-fold. The binding stoichiometry was 1 mol of TNP nucleotide per mole of monomer T antigen. The binding of TNP-ATP was more temperature dependent than that of TNP-ADP. The enthalpy change contributed nearly half of the energy for TNP-ATP binding, whereas binding of TNP-ADP was primarily entropy driven. Both TNP-ATP and TNP-ADP were strong inhibitors of the T antigen ATPase activity, confirming the high affinities of the TNP nucleotides for the ATP binding site. Like the parent nucleotides, they also induced T antigen hexamer formation. Using the TNP nucleotides as fluorescent probes, we have measured the affinity of various nucleotides and analogues for T antigen. The results indicate that the nucleotide binding specificity of T antigen was similar to that of the prokaryotic helicases Dna B and Rep, suggesting closely related ATP binding sites in the three DNA helicases.

T antigen, a multifunctional protein encoded by the early region of the simian virus 40 (SV40)[1] genome, directs the initiation of viral DNA replication (reviewed in *1*). It consists of 708 amino acids, with a calculated molecular weight of 82 500, and serves 3 major functions in SV40 DNA replication. It binds to specific DNA sequences in the viral origin of DNA replication, it recruits cellular replication proteins to the viral origin, and it unwinds the two parental strands of the viral DNA to allow replication.

To perform these functions, T antigen is equipped with an ATPase (*2*) and a 3′−5′ DNA helicase activity (*3*), as well as the ability to interact specifically with SV40 DNA (*2*) and cellular replication proteins (*4*−*8*). Several studies have indicated that the ATPase activity resides in the C-terminal half of T antigen between residues 418 and 616, whereas the helicase activity requires residues 131−627 (*9*−*11*). T antigen, upon binding to ATP or ADP in the presence of Mg^{2+}, assembles into hexamers (*12, 13*). Single hexamers of T antigen have DNA helicase activity at replication forks (*14*−*16*). However, in the presence of ATP or ADP and DNA carrying the SV40 core origin sequence, a double hexamer structure arises that is thought to encircle the DNA and to be responsible for bidirectional unwinding of the parental DNA (*12, 13, 15, 17*−*20*). Since the interactions of T antigen with ATP and ADP play a central role in the assembly of T antigen hexamers and ATP-dependent unwinding of SV40 DNA, characterization of the ATP binding site of T antigen should provide a foundation to learn how ATP binding and hydrolysis are coupled to T antigen double hexamer assembly on viral origin DNA, DNA unwinding, and helicase translocation. Studies of ATP binding and hydrolysis by equilibrium and kinetic measurements have been reported for several prokaryotic replicative helicases, such as the Dna B helicase (*21*−*23*), and the Rep helicase (*24*−*26*). Although T antigen is one of the better characterized eukaryotic helicases to date, to our best knowledge such information is not yet available for T antigen.

Fluorimetry offers an attractive approach for characterization of protein−ligand interactions (*27*−*29*) and in particular for helicase−nucleotide interactions (reviewed in *30*). In this work, we have employed fluorescent 3′(2′)-*O*-(2,4,6-trinitrophenyl)adenine nucleotide analogues (TNP-adenine nucleotides) to investigate the ATP binding site in T antigen.

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[1] Abbreviations: TNP nucleotide, 3′(2′)-*O*-(2,4,6-trinitrophenyl)-adenine nucleotide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SV40, simian virus 40.

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The TNP nucleotides have been previously utilized as fluorescent probes for various ATPase systems, such as chloroplast coupling factor 1 (*31, 32*), mitochondrial F1-ATPase (*33, 34*), sarcoplasmic reticulum Ca^{2+}-ATPase (*35, 36*), Na,K-ATPase (*37, 38*), Dna B helicase (*21, 22*), and cystic fibrosis transmembrane regulator (*39*). We demonstrate here that TNP nucleotide binding to T antigen induces a strong increase in the fluorescence of the TNP nucleotides and quenching of the protein tryptophan fluorescence, indicating that these nucleotides may serve as sensitive and specific probes for the nucleotide binding site in T antigen. In the accompanying paper, we demonstrate the utility of TNP nucleotides to study the interactions of T antigen with viral DNA and cellular replication proteins in solution.

MATERIALS AND METHODS

Nucleotides were obtained from Boehringer Mannheim. The fluorescent nucleotide analogues, 3′(2′)-*O*-(2,4,6-trinitrophenyl)-ATP and 3′(2′)-*O*-(2,4,6-trinitrophenyl)-ADP, were purchased from Molecular Probes (Eugene, OR).

Proteins. T antigen (wild-type) was purified from baculovirus-infected insect cells by immunoaffinity chromatography (*17*), and purity was verified by SDS−polyacrylamide gel electrophoresis. Protein concentrations were determined by the UV absorption at 280 nm assuming a calculated extinction coefficient (ϵ) of 66 000 M^{-1} cm^{-1} for T antigen (*17*).

Fluorescence Measurements. Fluorescence measurements were performed on a Perkin-Elmer MPF-44A fluorescence spectrophotometer. The slits were usually set at 6 nm. Fluorescence titrations were monitored either at $\lambda_{exc} = 300$ nm, $\lambda_{em} = 345$ nm (protein tryptophan fluorescence) or at $\lambda_{exc} = 415$ nm, $\lambda_{em} = 547$ nm (TNP fluorescence). The temperature of the sample holder and hence the cuvette was maintained at room temperature (20 °C) or otherwise controlled by circulating water through a thermostat (±0.1 °C). The TNP nucleotide was delivered from a stock solution with an Eppendorf micropipet. All titration points were corrected (*22*) for dilution (<4%) and the inner filter effect according to

$$F = F_{obsd}(V_i/V_o)10^{0.5\epsilon cl}$$

where F and F_{obsd} are the corrected and measured fluorescence intensities in arbitrary units; the term V_i/V_o accounts for the dilution at the ith addition of the nucleotide analogue; the inner filter effects due to nucleotide absorption were corrected according to Beer−Lambert's law, assuming linear absorption, where ϵ is the sum of molar absorbance at the wavelengths of interest, c is the added TNP nucleotide concentration (M), and l is the length of the light path (0.5 cm).

The fluorescence due to unspecific binding was measured in the presence of excess (2.6 mM) ATP. This value was subtracted from the total fluorescence, thus yielding the specific fluorescence enhancement (ΔF). All titrations were repeated at least 2 times. The data were fitted with MicroCal Origin program by nonlinear regression analysis.

ATPase Activity Measurements. The ATPase activity of T antigen was measured by incubating 0.34 μM T antigen with 0−100 mM ATP and 0.4 mCi of [γ-^{32}P]ATP (ICN, 3000 Ci/mmol) in 50 mM Tris-HCl, pH 8, 10 mM NaCl, 7 mM $MgCl_2$, 0.05% NP-40, and 1 mM DTT. For inhibition studies, the indicated amount of the competitor nucleotide was also included. After 10 min at 20 °C, 1 μL was spotted onto PEI-cellulose F thin-layer chromatography plates (Merck), and the plates were developed in 0.75 M NaH_2PO_4. Released phosphate (P_i) was quantitated with a phosphorimager.

Hydrolysis of TNP-ATP by T Antigen ATPase. A 50 μL solution containing 700 μM TNP-ATP and 2.1 μM T antigen in 20 mM Hepes, 5 mM $MgCl_2$, pH 8.0, was incubated at room temperature and 37 °C for up to 60 min. At time intervals, 10 μL of reaction mixture was removed and the reaction stopped by adding 17% trichloroacetic acid. An aliquot of 2 μL was applied on a silica 60 gel plate (with fluorescent indicator) and developed with a solvent mixture consisting of dioxane, 2-propanol, 11 N NH_4OH, and H_2O (40:22:36:34 by volume) (*40*). No hydrolysis of TNP-ATP was detected under these conditions.

Hexamer Formation of T Antigen in the Presence of Nucleotides. Hexamer formation was assayed as described (*41*). Briefly, 2.3 μM T antigen was incubated in a 10 μL reaction with 1 mM of the stated nucleotide for 30 min at 37 °C in 30 mM Hepes−KOH, pH 7.8, 7 mM magnesium acetate, 1 mM DTT. Then glutaraldehyde was added at a concentration of 0.2%. Incubation was continued for a further 5 min, and complexes were analyzed by native gel electrophoresis on a 5−15% gradient gel (BioRad, Munich) using a Tris/glycine buffer (25 mM Tris; 190 mM glycine). After transfer to nitrocellulose, proteins were detected with T antigen-specific monoclonal antibodies using the ECL detection system from Amersham.

RESULTS

Binding of TNP Nucleotides to T Antigen As Revealed by Fluorescence Measurements. The fluorescence of TNP-ATP and TNP-ADP was substantially enhanced upon their addition to T antigen. As shown in Figure 1A, we recorded the fluorescence emission of TNP-ATP with the excitation wavelength set at 415 nm. In the buffer alone, the fluorescence emission was centered maximally at 550 nm for TNP-ATP (spectra not shown). T antigen alone displayed little fluorescence at these wavelengths; however, in the presence of T antigen, the fluorescence of TNP-ATP increased, and the maximal emission wavelength was shifted to 539 nm ($\Delta\lambda = \lambda_{bound} - \lambda_{buffer} = -11$ nm), indicative of a hydrophobic ATP binding site in the protein. A similar enhancement of fluorescence was observed using TNP-ADP.

At room temperature, the fluorescence increased rapidly to a maximum upon addition of TNP-ATP to T antigen. With some T antigen preparations, we observed a subsequent slow decrease in the fluorescence intensity. At lower TNP-ATP concentrations, this secondary fluorescence decrease was faster. The amplitude change amounted to up to 6% of the total fluorescence, depending on the concentration of the added TNP-ATP. This slow decrease was not due to hydrolysis of the fluorescent ATP derivative by the ATPase, as shown by thin-layer chromatography. In fact, the same slow decrease was observed with TNP-ADP. The basis for this slow fluorescence decrease remains unknown.

When a large excess of ATP (>200-fold) was added, the fluorescence was decreased rapidly, restoring the intensity

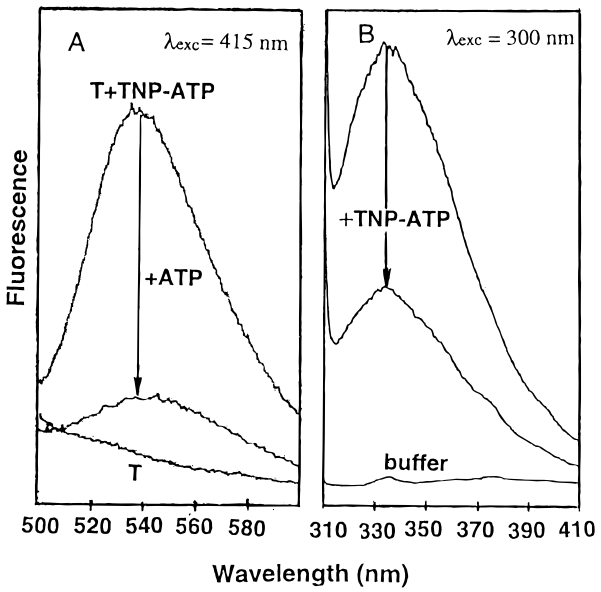

FIGURE 1: Fluorescence spectra of TNP-ATP bound to T antigen. (A) Emission spectra of 1.0 μM TNP-ATP fluorescence in the presence of 1.5 μM T antigen. The buffer contained 20 mM Hepes, 5 mM $MgCl_2$, pH 8.0. Fluorescence excitation was at 415 nm. After the recording, excess (2.6 mM) ATP was added to displace the bound TNP-ATP from the binding sites. (B) Emission spectra of intrinsic fluorescence of 1.0 μM T antigen in the presence of 5 μM TNP-ATP in the same buffer. Fluorescence excitation was at 300 nm.

and maximal emission wavelength to values comparable to those in the buffer (Figure 1A). The fluorescence (F_{total}) upon TNP nucleotide addition reflected the contributions both from the TNP nucleotide bound to the protein and from the free TNP nucleotide in buffer. As a large excess of ATP should displace all the bound TNP nucleotide from the ATP binding site, the difference (ΔF) between the total fluorescence and the fluorescence in the presence of excess ATP should represent the fluorescence enhancement due to specific binding to the ATP binding site. This result indicates that TNP-ATP bound to the same site as ATP.

We also investigated the quenching of the protein tryptophan fluorescence by the TNP nucleotides. Figure 1B presents the emission spectra of the T antigen tryptophan fluorescence (λ_{exc} = 300 nm) before and after addition of TNP-ATP. In the absence of the nucleotide, the spectrum showed an emission maximum at 336 nm, suggesting a moderate burial of the tryptophans in the protein (*42*). When the TNP nucleotide was present, it quenched the tryptophan fluorescence, probably due to resonance energy transfer to the bound TNP nucleotide. Although about half of the tryptophan fluorescence was quenched under these conditions, the maximal emission wavelength was not altered. The quenching of tryptophan fluorescence occurred rapidly on adding TNP-ATP or TNP-ADP; we observed no secondary slow phase of fluorescence change. In contrast with TNP-ATP and TNP-ADP, the nucleotides ATP, ATPγS, and ADP quenched the tryptophan fluorescence too little (<2%) to allow quantitation of the binding and therefore were not further investigated.

Figure 2A shows typical fluorescence titrations of T antigen with TNP-ADP where the TNP emission fluorescence (λ_{exc} = 415 nm, λ_{em} = 547 nm) was measured. The fluorescence was corrected for dilution and inner filter effects (see Materials and Methods). A plot of the specific fluorescence change (ΔF) versus TNP-ADP concentration gave a saturation curve. The maximal fluorescence enhancement (ΔF_{max}) and the dissociation constants K_D were obtained by data fitting (*29*). To confirm the linear concentration dependence of the maximum fluorescence enhancement (ΔF_{max}), the T antigen concentration was varied 20-fold from 0.3 to 6 μM. Figure 2B summarizes the ΔF_{max} and K_D values obtained for each T antigen concentration. The ΔF_{max} increases linearly with T antigen concentration, and therefore the fluorescence yield ($\phi = \Delta F_{max}/[T]$) remains constant irrespective of the T antigen concentration, suggesting that the bound nucleotide concentration is proportional to the specific fluorescence intensity. The K_D (1.6 $\pm$ 0.2 μM) appears to be independent of the T antigen concentration.

The stoichiometry of ligand−T antigen interaction was evaluated in a separate titration (Figure 3). A fluorescence yield of ϕ = 13.2 μM^{-1} was calculated by using the protein concentration. This yield represents the specific fluorescence enhancement when 1 μM T antigen was fully saturated with TNP-ADP. The fluorescence yield (ϕ) was also estimated from the titration of 1 μM TNP-ADP with increasing T antigen concentration. We found that this procedure gave an equal value of fluorescence yield, indicating that the binding stoichiometry was 1 mol of TNP-ADP to 1 mol of T antigen monomer.

The binding stoichiometry was also obtained by replotting the titration data of Figure 3A as a mass action plot (Figure 3B) according to (*43*)

$$r/[L]_{free} = n/K_D - r/K_D \quad (1)$$

where r is the ratio of the bound ligand concentration to the T antigen concentration, $[L]_{free}$ the concentration of free ligand, n the binding stoichiometry (moles of TNP-ADP per mole of monomeric T antigen), and K_D the dissociation constant. The concentration of bound ligand was estimated from the micromolar fluorescence enhancement according to $[nucleotide]_{bound} = \Delta F/\phi$. The concentration of free ligand was calculated from $[L]_{free} = [nucleotide]_{total} - [nucleotide]_{bound}$. The data fitted well to a straight line. From the intercept on the x-axis, we estimated n = 0.98 $\pm$ 0.03, indicating a binding stoichiometry of 1 mol of TNP-ADP to 1 mol of T antigen. From the slope, a dissociation constant K_D of 2.6 $\pm$ 0.1 μM was obtained. The Hill plot (*28*) revealed a coefficient n of 1.0, suggesting no cooperativity in ligand binding.

As shown above by fluorescence titrations, the fluorescence of TNP-ATP increased by 8-fold on binding to T antigen, relative to the fluorescence in buffer. This result can be compared to fluorescence measurements in 90% dioxane, where the fluorescence increased 13-fold with a concomitant blueshift ($\Delta\lambda$) of −16 nm. These data provide further evidence for the hydrophobic nature of the ATP binding site.

Nucleotide binding was also quantitatively evaluated by the quenching of the tryptophan fluorescence of T antigen (λ_{exc} = 300 nm, λ_{em} = 345 nm). TNP-ADP quenched the fluorescence increasingly as the nucleotide concentration increased (Figure 3C). The double reciprocal plot was linear and showed that TNP-ADP could quench up to 56% of the total tryptophan fluorescence of T antigen. The mass action

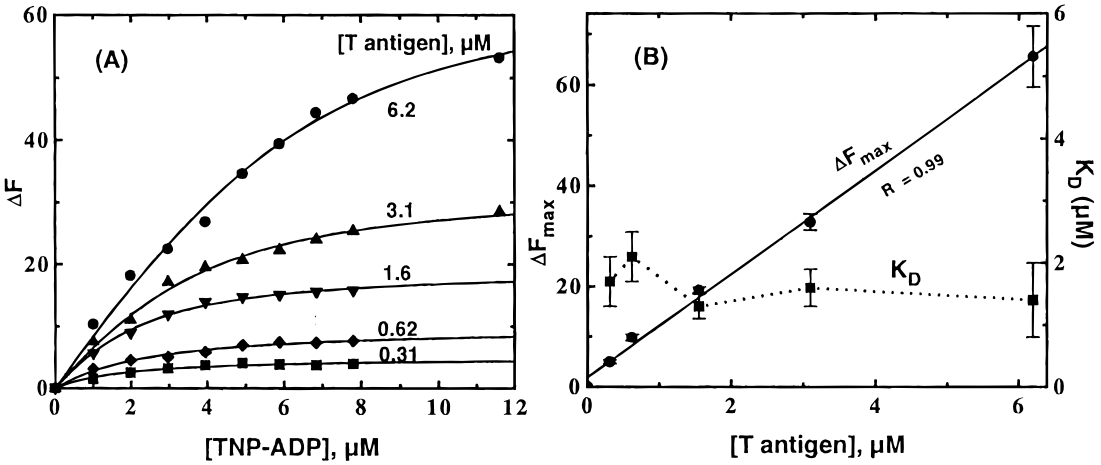

FIGURE 2: Maximum fluorescence enhancement (ΔF_{max}) and dissociation constant (K_D) dependence on T antigen concentration. (A) Fluorescence titrations were performed in 0.31−6.2 μM T antigen at pH 8.8 with TNP-ADP. Titration curves were fitted according to Huang and Klingenberg (*29*). (B) Plot of ΔF_{max} and K_D versus T antigen concentration.

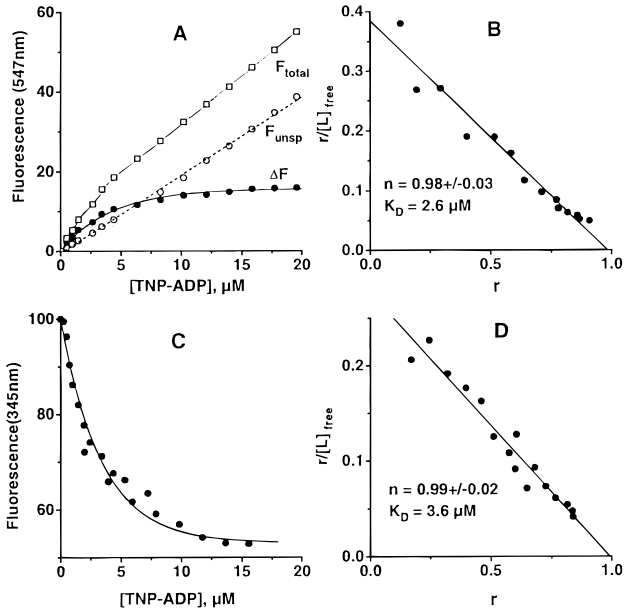

FIGURE 3: Titration of T antigen with TNP-ADP. Fluorescence titration of 1.4 μM T antigen with TNP-ADP in 20 mM triethylamine, 2.5 mM potassium phosphate, 20% glycerol, and 5 mM $MgCl_2$, pH 8.8. Fluorescence monitored at $\lambda_{exc} = 415$ nm, $\lambda_{em} = 547$ nm (TNP nucleotide fluorescence). (A) Fluorescence increase versus TNP-ADP concentration. F_{total} and F_{unsp} are the measured fluorescence intensities (arbitrary units) on addition of TNP-ADP to the T antigen and to the T antigen preincubated with 2.6 mM ATP, respectively. (B) mass action plot of (A) according to eq 1. The straight line is a least-squares fit with $K_D = 2.6$ μM and $n = 0.98$. Fluorescence monitored at $\lambda_{exc} = 300$ nm, $\lambda_{em} = 345$ nm (tryptophan fluorescence). (C) Fluorescence quenching versus TNP-ADP concentration: the double reciprocal plot reveals a maximal quenching of 56.1% of the total protein fluorescence. (D) Mass action plot of (C) using eq 1. The straight line is a least-squares fit with $K_D = 3.6$ μM and $n = 0.99$.

plot (Figure 3D) revealed a 1:1 binding stoichiometry with $K_D = 3.6 \pm 0.2$ μM. The Hill plot again suggested no cooperativity ($n = 1.05 \pm 0.09$) in ligand binding. The nucleotide binding parameters are given in Table 1.

The pH dependence of binding (data not shown) was weak between pH 7.5 and 8.8. The K_D for TNP-ATP was 0.2−0.5 μM, and was 4−8-fold lower than for TNP-ADP. In all cases, the binding stoichiometry was 1 mol of TNP nucleotide per mole of T antigen, and binding occurred without cooperativity (Hill coefficient $n \approx 1$). The results obtained by measuring the emission fluorescence of the TNP nucleotide were in good agreement with those determined by quenching of the tryptophan fluorescence (Table 1).

Influence of Mg^{2+} and Salt on TNP Nucleotide Binding. The fluorescence measurements reported above were conducted in the presence of 5 mM Mg^{2+}. As Mg^{2+} was required for the ATPase activity of T antigen (*44*), we investigated the influence of Mg^{2+} on TNP-ATP binding to T antigen. In the absence of Mg^{2+}, TNP nucleotide binding to T antigen was still detected; however, the apparent specific fluorescence enhancement ΔF was 2−4-fold lower than in the presence of 5 mM Mg^{2+} (figure not shown). This lower fluorescence enhancement could be explained by (1) lower binding, but equal maximal fluorescence enhancement (ΔF_{max}), (2) equal binding, but lower ΔF_{max}, or (3) a mixture of the two factors. These possibilities were resolved by fluorescence titrations. For TNP-ATP at pH 8.0 in the absence of Mg^{2+}, we measured a K_D of 2.6 μM and a ΔF_{max} of 6.0 (arbitrary units), whereas in the presence of 5 mM Mg^{2+} the K_D and ΔF_{max} were 0.55 μM and 6.1, respectively. These results demonstrate that the lower fluorescence response in the absence of Mg^{2+} was due to the decreased affinity of TNP nucleotides for T antigen. A similar observation was reported by Moore and Lohman (*45*), where binding of 3′(2′)-*O*-(*N*-methylanthraniloyl)-ATP to the prokaryotic Rep helicase was detectable in the absence of Mg^{2+}, albeit with substantially lower affinity than in the presence of Mg^{2+}.

Figure 4 illustrates the effects of sodium chloride and sodium phosphate on TNP nucleotide binding to T antigen at pH 7.5. Double reciprocal plots showed a competitive inhibition pattern, indicating that the anions may bind to the ATP binding site but be displaced by the TNP nucleotide. The inhibitory effect of salt can be evaluated from the equation (*43*):

$$K_i = [I]/(K'_D/K_D - 1) \quad (2)$$

where K_i is the inhibition constant, [I] is the concentration of the inhibitor or here of the salt, and K'_D and K_D are the dissociation constants of the TNP nucleotide in the presence and absence of the salt, respectively. The calculated inhibi-

Table 1: Parameters of TNP-ATP and TNP-ADP Binding to the Wild-Type T Antigen[a]

	TNP fluorescence			fluorescence quenching	
	TNP-ADP	TNP-ATP		TNP-ADP	TNP-ATP
K_D (μM)	2.6 ± 0.1	0.35 ± 0.03	K_D (μM)	3.6 ± 0.2	0.44 ± 0.05
stoich *n*	0.98 ± 0.03	0.89 ± 0.05	stoich *n*	0.99 ± 0.02	0.80 ± 0.01
Hill *n*	1.0	1.0 ± 0.04	Hill *n*	1.0 ± 0.1	1.0 ± 0.04
ϕ_b/ϕ_{H_2O}	8.8	8.7	Q_{max}	0.56	0.50

[a] Fluorescence titration of T antigen was performed in buffer containing 20 mM Hepes, 5 mM $MgCl_2$, pH 8.8. K_D is the dissociation constant, stoich *n* is the binding stoichiometry (moles of TNP nucleotide per mole of monomeric T antigen). Hill *n* is the Hill coefficient. ϕ_b/ϕ_{H_2O} is the ratio of the fluorescence yield of bound TNP nucleotide ($\lambda_{exc} = 415$ nm, $\lambda_{em} = 547$ nm) to that in the aqueous buffer. Q_{max} is the maximal quenching of the protein tryptophan fluorescence ($\lambda_{exc} = 300$ nm, $\lambda_{em} = 345$ mm).

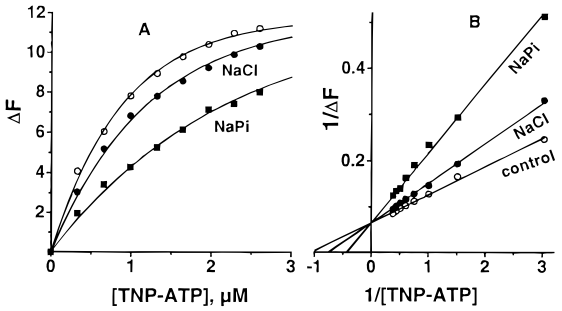

FIGURE 4: Influence of salt on TNP-ATP binding to T antigen. Fluorescence titration of 1.7 μM T antigen in the absence and presence of 50 mM sodium chloride and 50 mM sodium phosphate (pH 7.5). (A) Titration curves in the presence and absence of salt. (B) Double reciprocal plots. The K_D was 1.0 μM in the absence of the salt, and 1.5 and 2.6 μM in the presence of chloride and phosphate, respectively.

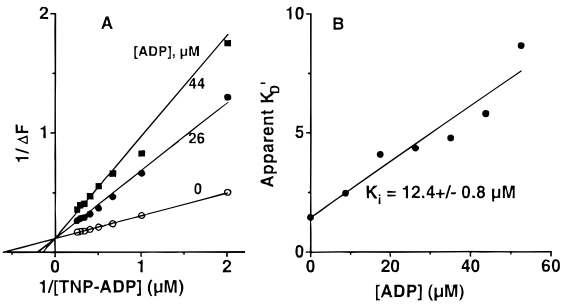

FIGURE 5: Competitive inhibition by ADP of TNP-ADP binding to T antigen. Fluorescence titration of 1.1 μM T antigen (pH 7.5). ADP was incubated with the T antigen for 15 min prior to titration. (A) Double reciprocal plot; (B) theoretical fitting of the concentration dependence of the apparent K'_D with eq 2.

tion constants (K_i) were 100 mM for sodium chloride and 30 mM for sodium phosphate.

Nucleotide Binding Specificity of T Antigen. The binding affinity of various nucleotides and analogues to T antigen was measured by using TNP nucleotides as a probe. The nucleotide of interest was first incubated with T antigen for 15 min, and then increasing concentrations of TNP nucleotide were titrated into the mixture. Figure 5A shows the double reciprocal plots of fluorescence titrations with TNP-ADP added to T antigen in the presence of ADP. Competitive inhibition by ADP was observed, further confirming that the TNP nucleotide and ADP bind to the same nucleotide binding site. From the double reciprocal plots, we estimated the apparent dissociation constant K'_D at each concentration of ADP. A theoretical fitting of the concentration dependence of K'_D yielded a K_i of 12.4 ± 0.8 μM for ADP (Figure 5B). This value should correspond to the dissociation constant K_D of ADP binding to T antigen.

Table 2: Dissociation Constants (K_D) of T Antigen Complexes with Some Nucleotides and Analogues As Measured by Competition with TNP Nucleotides (pH 7.5)[a]

ligand	T antigen	Dna B	Rep
ATP		2.8	0.18
ATPγS	0.40 ± 0.20	0.10	0.45
AMPPNP	53 ± 1	1200	6.0
AMPPCP	73 ± 4		
ADP	12.4 ± 0.8	14	3.6
AMP	2600 ± 200		1000
GDP	65 ± 4		
UDP	750 ± 30		

[a] In all cases, competitive inhibition was observed; therefore eq 2 was used to calculate K_D (μM). The results were similar with either TNP-ATP or TNP-ADP as the competitor. The K_D values for the Dna B and Rep helicases determined by Kornberg and co-workers (*46, 47*) are listed for comparison.

Using the same procedure, we estimated the K_D values for binding of other nucleotides and analogues to T antigen, and the data are summarized in Table 2. The results obtained using TNP-ATP and TNP-ADP as the fluorescent probes were in good agreement. As shown in Table 2, the affinity of ATPγS (0.4 μM) was 150-fold higher than that of AMPPNP or AMPPCP. Among the nucleoside diphosphates, T antigen preferentially bound ADP, as the affinities of GDP and UDP were 5 and 60 times weaker. The affinity of AMP for T antigen was extremely weak. For comparison, we have also listed the data for the Dna B and the Rep helicases measured by the gel filtration method (*46, 47*). A comparison of the relative nucleotide affinities of the three replicative helicases reveals a similar nucleotide binding specificity.

Inhibition of ATPase Activity by TNP Nucleotides. To independently confirm the measured affinities of the nucleotide analogues for the ATP binding site in T antigen, we determined their inhibitory effects on its ATPase activity. Figure 6 shows the inhibition of the T antigen ATPase activity by several nucleotide ligands as a function of their concentrations. The inhibition can be best fitted (solid lines) by assuming a reversible binding of the ligands to the ATPase site. From these curves, we estimated the concentrations required for half-inhibition (K_i), which were 2.2 ± 0.3, 0.90 ± 0.01, 0.60 ± 0.19, 37 ± 5, and 113 ± 17 μM for TNP-ADP, ATPγS, TNP-ATP, ADP, and AMPPNP, respectively. These K_i values agree well with the corresponding dissociation constants (K_D) determined by fluorescence titrations (Tables 1 and 2). These inhibition data confirm the affinity of the various nucleotides for T antigen determined with the fluorescence method.

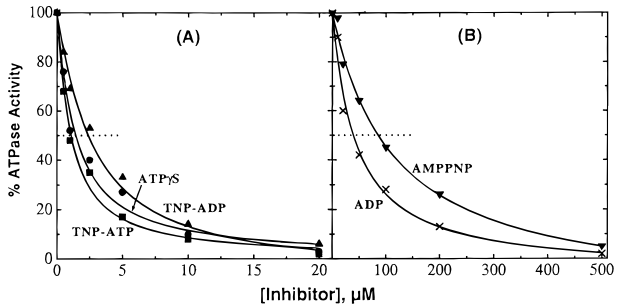

FIGURE 6: Inhibition of ATPase activity by various nucleotide analogues. ATPase assays in the presence of 10 mM ATP and the indicated competitor nucleotide concentration were carried out at 20 °C. The percent ATPase activity was plotted as a function of the competitor concentration. The K_i value (see text) corresponds to the competitor concentration that led to a decrease of 50% (dashed lines) in activity.

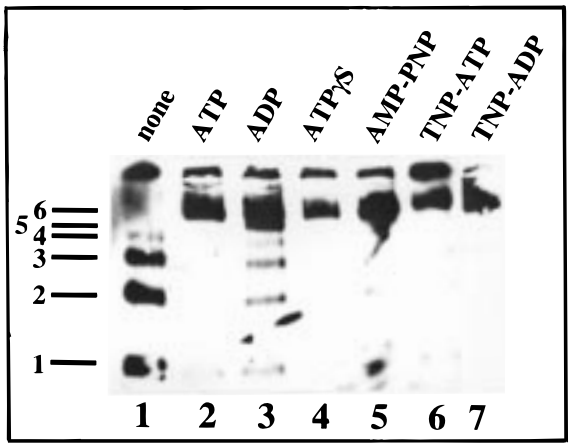

FIGURE 7: Hexamer formation in the presence of nucleotides and analogues. T antigen was incubated in the absence (lane 1) or presence of 1 mM of the indicated nucleotides (lanes 2−7). After cross-linking, complexes were resolved by native gel electrophoresis. The numbers on the left margin designate the oligomeric state of T antigen.

Binding of TNP Nucleotides to T Antigen Induces Hexamer Formation. To investigate whether the TNP nucleotide analogues were able to induce T antigen hexamer formation, T antigen was incubated in the presence of magnesium ions with the various nucleotides and then analyzed by native gel electrophoresis. As shown in Figure 7, T antigen in the absence of added nucleotide existed mostly as a monomer, with small amounts of dimer and trimer and trace amounts of tetramer (lane 1). TNP-ATP and TNP-ADP, like the other ligands ATP, ATPγS, AMPPNP, and ADP, induced hexamer formation (lanes 2−7). This result clearly shows that the addition of the bulky TNP group to the nucleotide apparently did not interfere with the correct intermolecular T antigen interactions in hexamer assembly.

Influence of Temperature on TNP Nucleotide Binding to T Antigen. We investigated the thermodynamics of TNP nucleotide binding (Table 3). From fluorescence titrations, we measured the dissociation constant (K_D) and maximal fluorescence enhancement (ΔF_{max}) at temperatures ranging from 3.8 to 34 °C. As the temperature increased from 3.8 to 34 °C, the K_D for TNP-ATP binding increased almost 3-fold (data not shown). The van't Hoff plot was a straight line, from which the enthalpy change was determined to be -15.2 ± 2.0 kJ/mol (Table 3). The free energy change was

Table 3: Thermodynamic Parameters of TNP Nucleotides Binding to T Antigen[a]

	TNP-ATP	TNP-ADP
ΔH (kJ/mol)	-15.2 ± 2.0	4.5 ± 1.6
ΔG (kJ/mol)	−33.0	−31.2
ΔS (J deg^{-1} mol^{-1})	61.2	123

[a] The dissociation constants at various temperatures were determined from fluorescence titrations in 20 mM Hepes, 5 mM $MgCl_2$, pH 8.0. The enthalpy change (ΔH) was evaluated from the van't Hoff plots. The free energy change ΔG (20 °C) was calculated from the dissociation constant according to $\Delta G = 2.303RT \log K_D$, where R is the gas constant and T the absolute temperature (K). The entropy change ΔS at 20 °C was calculated from the formula $\Delta S = (\Delta H - \Delta G)/T$.

−33.0 kJ/mol as calculated from the dissociation constant at 20 °C. These data indicated that half of the binding energy came from the enthalpy change. A entropy change of 61.2 J deg^{-1} mole^{-1} was obtained from the equation $\Delta S = (\Delta H - \Delta G)/T$. In contrast, TNP-ADP binding was a slightly endothermic process with a much weaker temperature dependence. The enthalpy change was measured to be 4.5 $\pm$ 1.6 kJ/mol (Table 3). From the K_D, we calculated a free energy change of −31.3 kJ/mol (20 °C). The entropy change was 2-fold higher (123 J deg^{-1} mole^{-1}), and was the primary driving force in the binding reaction. The apparent thermodynamic data for the binding of TNP-ATP and TNP-ADP to T antigen are listed in Table 3.

At lower temperatures, the fluorescence enhancement (ΔF_{max}) and consequently the fluorescence yield (ϕ) were higher for both TNP-ATP and TNP-ADP. As the fluorescence is sensitive to the microenvironment where the fluorescent group resides, we investigated whether the different fluorescence yields might correspond to different conformations of T antigen complexed with the TNP nucleotide, or whether they might arise simply from the physical properties of the fluorescent nucleotide. To distinguish between these possibilities, we measured the fluorescence of the free TNP nucleotides at the various temperatures in 90% dioxane. The results show that the fluorescence intensity decreased with temperature, coincident with the observed changes in the fluorescence yield (ϕ). This observation suggests that the higher ΔF_{max} at lower temperatures was not due to a conformational difference in the T antigen bound with the TNP nucleotide, but rather to the fluorescence properties of the TNP nucleotide per se.

DISCUSSION

TNP Nucleotides Are Suitable Probes To Study ATP Binding to T Antigen. ATP binding to T antigen is a key step in the initiation of SV40 DNA replication (reviewed in *1*; *8*, *20*). ATP plays a regulatory role in inducing conformational changes in T antigen that foster its assembly as a multimeric complex on the viral origin of DNA replication (*41*). Cycles of ATP hydrolysis play a catalytic role in the separation of complementary parental DNA strands and in the movement of T antigen along the DNA strand to which it is bound during replication (*3*, *20*). To better explore the molecular mechanisms of these events, we have studied the nucleotide binding properties of T antigen by using the fluorescent TNP nucleotide analogues. Since these analogues were able to induce T antigen hexamer formation (Figure 7) and competitively inhibit its ATPase activity (Figure 6),

they are potentially useful in the study of the influence of nucleotide binding on the various activities of T antigen, such as DNA binding, ATPase and helicase, and interaction with cellular replication proteins.

The fluorescence of the TNP nucleotides changed upon interaction with T antigen (Figures 1 and 2). Both the increased fluorescence of TNP-ATP and TNP-ADP upon binding to T antigen and the quenching of the neighboring tryptophans in T antigen were utilized as signals to measure the nucleotide binding of T antigen. Affinities of TNP-ATP and TNP-ADP were high, with association constants in the range of 10^6 M^{-1}. The dissociation constants determined by fluorescence titrations (Tables 1 and 2) agreed with the concentration required to inhibit half of the T antigen ATPase activity (K_i) (Figure 6). The affinity of TNP-ATP was 4−8-fold higher than that of TNP-ADP. That the dissociation constant (K_D) was independent of the T antigen concentration (Figure 2) may suggest that the fast titration procedure measured either only the monomeric T antigen−TNP nucleotide complex (i.e., prior to the hexamer formation step) or a mixture of monomeric and hexameric complexes provided that hexamer formation occurs without altering the affinity of the fluorescent nucleotide analogue.

We were unable to directly measure the affinity of ATP for T antigen, as the nucleoside triphosphate would be hydrolyzed by the ATPase. However, the affinity of ATP for the Dna B and Rep helicases was known, since they hydrolyze ATP poorly in the absence of DNA (*46, 47*). ATP and ATPγS displayed a similar affinity for the prokaryotic helicases, which was much higher than that of AMPPNP (Table 2). By analogy, we would predict that the intrinsic affinity of ATP for T antigen would be similar to that of ATPγS, but so far no data are available for a direct comparison. The K_m values for ATP reported for the T antigen ATPase and helicase reactions ranged from 0.5 to 4 μM (*20* and references cited therein). Theoretically this value sets the upper limit for the K_D. Thus, it appears that in contrast with ADP, TNP substitution enhanced the affinity of ATP for T antigen very little (about 2-fold), whereas it enhanced the affinity of ADP almost 11-fold.

We have also attempted to use 2′-*O*-dansyl nucleotides and 3′(2′)-*O*-(5-dimethylaminonaphthalene-1-carbonyl) nucleotides to investigate the nucleotide binding site in T antigen. These fluorescent nucleotide analogues were shown to bind with highly enhanced fluorescence to the mitochondrial uncoupling protein (*29*) and to the ADP/ATP carrier (*28*). However, our results show that the substitution with the bulkier dimethylaminonaphthalene group could not be accommodated by the binding site of T antigen. These data suggest that the nucleotide binding site in T antigen is more specific for the ligand.

The nucleotide binding site displayed little pH dependence between pH 7.5 and 8.8, in contrast with the strong pH dependence of nucleotide binding in the mitochondrial uncoupling protein (*29*). Also the inhibition of nucleotide binding by anions was much weaker for T antigen than for the uncoupling protein. These differences may reflect two types of nucleotide binding sites: one in the ATPase which generally requires Mg^{2+} for binding and catalysis, and the other in the uncoupling protein where the naked phosphate moiety participates in nucleotide binding, undergoing protonation/deprotonation at the terminal phosphate and at the binding center as a regulatory mechanism for the uncoupling protein (*29, 48*).

We observed a striking difference in the thermodynamics of TNP-ATP and TNP-ADP binding to T antigen (Table 3). The binding of TNP-ADP was primarily driven by entropy change, suggesting that the binding was accompanied with strong hydrophobic interactions and removal of water from the binding site (*49*). A stronger hydrophobic interaction for TNP-ADP was also implied by the much higher enhancement of the ADP affinity by TNP substitution. However, the binding of TNP-ATP was more temperature dependent with an enthalpy change amounting to half of the total free energy change (Table 3). Apart from hydrophobic interactions, which were apparently weaker than in the case of TNP-ADP, the binding of TNP-ATP was driven partially by release of heat.

Comparison of T Antigen with the Dna B Helicase. The Dna B protein is a hexameric replicative DNA helicase found in *E. coli* (recently reviewed in *30*; *50*). A comparison of the nucleotide binding specificities of these two helicases reveals close similarities (Table 2). A similar specificity was reported for the dimeric Rep helicase, although its nucleotide binding affinity was generally stronger. Like T antigen, which was unable to hydrolyze TNP-ATP in the absence of DNA, the Dna B ATPase hydrolyzed it extremely slowly in either the presence or the absence of DNA (*21, 22*).

Although the nucleotide binding specificity of T antigen resembled that of the Dna B protein, there were several quantitative differences. T antigen had a much higher (4−8-fold) affinity for TNP-ATP than TNP-ADP, whereas with the Dna B protein, the affinity of TNP-ATP was only double that of TNP-ADP. With T antigen, the fluorescence parameters (ϕ) were equal for both TNP-ATP and TNP-ADP, while the maximal fluorescence enhancement of TNP-ATP bound to Dna B protein was double that of TNP-ADP.

As shown in this work, T antigen bound TNP-ATP or TNP-ADP with a stoichiometry of 1 mol of TNP nucleotide per monomeric T antigen, whereas Biswas and co-workers (*21*) reported a stoichiometry of only 3 TNP nucleotides per hexameric Dna B protein. They interpreted their data as an indication that the lower stoichiometry was due to the bulky TNP group, which allowed the binding of only three nucleotides, as compared to six ATP molecules. However, in a later study using tryptophan fluorescence quenching, Bujalowski and Klonowska (*22*) measured a stoichiometry of 6 mol of TNP nucleotide per hexameric Dna B protein. They titrated up to a nucleotide concentration of 200 μM, and observed a hexagon-type binding isotherm; i.e., binding of the first three nucleotides occurred without cooperativity, whereas binding of the second three nucleotides was accompanied with strong negative cooperativity ($n = 0.55$ for TNP-ATP and 0.31 for TNP-ADP). These data may well explain the lower stoichiometry observed by Biswas and co-workers in titrations up to 10 μM TNP nucleotide, which presumably the measured binding of only the first three nucleotides.

We have also employed the approach of Bujalowski and Klonowska (*22*) to titrate the T antigen and observed a similar hexagon biphasic binding isotherm (figure not shown). However, the second phase occurred at a much lower nucleotide concentration than with Dna B helicase, suggesting a cooperativity much less negative for T antigen

than for Dna B. Thus, under the conditions presented here, we measured a stoichiometry of 6 mol of TNP nucleotide to 1 mol of hexameric T antigen without detectable cooperativity. At least one other hexameric helicase, the RNA-dependent Rho helicase, has been reported to display two classes of nucleotide binding sites, which are thought to be important for the directional helicase activity of the protein (*51*−*53*). Moreover, in the presence of DNA, the dimeric Rep helicase also displays negative cooperativity in nucleotide binding to its two sites, suggesting that Rep and the hexameric helicases may share similar mechanisms for helicase action (*24*, *25*, *30*).

Like T antigen, several prokaryotic helicases are known to interact physically and functionally with DNA polymerases and primases. For example, interactions between Dna B and the Tau subunit of DNA polymerase III at *E. coli* replication forks serve to accelerate DNA unwinding and coordinate replication of the leading and lagging strands (*54*−*56*). Furthermore, protein−protein interactions between Dna B and the *E. coli* primase control the synthesis of Okazaki fragments on the lagging strand (*57*). Similar interactions occur between the hexameric bacteriophage T4 helicase gp41 and primase (*58*), between gp41 and T4 DNA polymerase (*59*), and between the subunits of the T7 gene 4 helicase−primase hexamers. In the accompanying paper, we demonstrate the utility of TNP adenine nucleotide binding to T antigen as a probe to study new aspects of its interactions with human DNA polymerase α-primase, as well as with SV40 origin DNA (*60*).

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