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# Activation of ATPase Activity of Simian Virus 40 Large T Antigen by the Covalent Affinity Analog of ATP, Fluorosulfonylbenzoyl 5′-Adenosine

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**Fluorosulfonylbenzoyl 5′-adenosine (FSBA) bound to one site in simian virus 40 large T antigen (T) and covalently modified greater than 95% of the molecules in a complete reaction. This analog for ATP specifically cross-links to the Mg-phosphate pocket in ATP-binding sites. Cyanogen bromide cleavage and tryptic digestion of [$^{14}$C]FSBA-labeled protein, paired with T-specific monoclonal antibody analyses, were used to map the site in T to a tryptic peptide just C terminal to the PAb204 epitope. The location of the FSBA linkage was consistent with the predicted tertiary structure of the ATP-binding region in T described previously (M. K. Bradley, T. F. Smith, R. H. Lathrop, D. M. Livingston, and T. A. Webster, Proc. Natl. Acad. Sci. USA 84:4026–4030, 1987). Binding of FSBA to T was cooperative, implying an interaction between two binding sites. This could occur if the protein formed a dimer, and it is known that the ATPase activity is associated with a dimeric T. Most interesting was the activation of the ATPase when up to 50% of T was bound by the analog. The effect was also produced by preincubation with millimolar concentrations of ATP or the nonhydrolyzable analog γβ-methylene 5′-adenosine diphosphate at elevated temperatures. When greater than 50% of T was modified by FSBA, the ATPase was inhibited as the analog cross-linked to the second, previously activated, binding site. These data support a dual function for the one ATP-binding site in T as both regulatory and catalytic.**

It is known that the large T antigen (T) of simian virus 40 (SV40) has an intrinsic ATPase activity (4, 8), and the covalent affinity analog for ATP, fluorosulfonylbenzoyl 5′-adenosine (FSBA), was used as a probe to further investigate the ATP-binding site. FSBA has been used effectively for mapping the catalytic site for several well-known enzymes such as RecA (23) and cyclic AMP-dependent protein kinase (39). In FSBA, a nucleophilic group is substituted for the Mg-phosphoryl region of Mg-ATP, approximating its size and shape sufficiently well enough to bind and cross-link specifically to the Mg-phosphate pocket in ATP-binding sites (see structure in Fig. 1) (12).

The monkey papovavirus SV40 replicates its own DNA using infected host cell factors and one virally encoded gene product, T. T also has other regulatory roles in the infected host involving transcription, cellular DNA replication, and growth regulation associated with oncogenic transformation (for reviews, see references 16 and 30). For viral DNA replication, the N-terminal portion of T protein must bind specifically to the viral DNA at the origin (1, 36). A large part of the C-terminal region of the T protein is also essential for SV40 DNA replication activity. Several mutations mapped to that location encode temperature-sensitive gene products that are defective for both replication and oncogenic transformation (16, 25). It is apparently easy to disrupt the peptide by mutating that region, since only a few point mutants have been isolated that are defective in viral DNA replication but remain stable and retain transformation activity (e.g., at residues 509 and 522 [11, 26]). These mutant T proteins bind to the SV40 DNA origin of replication but are defective for the ATPase activity shown to be associated with the wild-type gene product. From these data, it appears that a T-associated ATPase is required for the replication function (8, 11, 26).

The ATP-binding site in SV40 T has been located in the C-terminal half of the protein by additional criteria. A prediction of the three-dimensional structure of the ATP-binding site in T has been presented, based on the known structure of the GTP-binding protein EF-Tu, the translation elongation factor of *Escherichia coli* (6). Included in this model is the region of the protein between residues 418 and 528 (out of 708), predicted to fold into a five-strand β-sheet with at least three of the central strands arranged in parallel. Residues in T that contact the α-phosphate, ribose, and Mg-phosphoryl groups of MgATP were proposed. Several sets of data support this model. First, monoclonal antibodies specific for the region of T near residue 455 effectively interfere with the ATPase activity (6, 8). Second, the covalent affinity analog 2′,3′-dialdehyde ATP (oATP) inhibits the ATPase activity of T and binds specifically to a cyanogen bromide (CNBr) fragment of T located between residues 413 and 528 (10). Third, adenylylation of purified T by incubation with unmodified MgATP was mapped to a single tryptic peptide within the same CNBr fragment of the protein (5). However, neither adenylylation nor covalent modification with oATP binds more than 50% of the T protein (5, 9; M. K. Bradley and P. Clertant, unpublished data).

Data presented here using the covalent affinity analog FSBA show that >90% of T can be labeled, that there is only one binding site for ATP in T, and that the binding site is located in the position proposed by our model. A single FSBA-labeled tryptic peptide was isolated and mapped to the binding site by using T-specific monoclonal antibodies. Most interestingly, the binding studies indicated the presence of two forms of T that bound the analog in that one site. One was ATPase active, and binding of FSBA specifically blocked the catalytic activity as shown for other enzymes. A second form of T was initially inactive as an ATPase, and binding of FSBA (or Mg-ATP or Mg-AMPPCP [AMPPCP is γβ-methylene 5′ adenosine diphosphate]) resulted in the

activation of T-ATPase activity. These results can be explained by a simple model involving an ATPase-active dimer (4). The binding of ATP to a monomer of T would be predicted to induce a conformational change favoring dimerization leading to enzyme activation. This effect has been shown for several other proteins having an ATP- or GTP-binding site predicted to have structural features similar to those of T (RecA, adenylate kinase, Ras, EF-Tu [6; M. K. Bradley and T. A. Webster, unpublished observations]). Therefore, the ATP-binding site in T has a regulatory as well as a catalytic role, and either role would be affected by mutations in the site leading to defects in viral DNA replication.

## MATERIALS AND METHODS

**Chemicals and reagents.** Stocks of nucleotides (Sigma Chemical Co.) were made at 100 mM, neutralized to pH 7.4 with NaOH, and stored at −20°C. FSBA (Sigma) was dissolved in dimethylsulfoxide (DMSO) to 100 mM and kept in the dark at room temperature. Exact concentrations of nucleotide used in the experiments were determined by UV spectroscopy; the extinction coefficient for ATP at $A_{259}$ was $14.5 \times 10^3$ and for FSBA at $A_{259}$, it was $13.5 \times 10^3$ (18).

Radioactively labeled nucleotides were purchased from Amersham Corp., including [$\gamma$-$^{32}$P]ATP (3,000 Ci/mmol) for use as a substrate in ATPase assays and custom synthesized [$^{14}$C]FSBA (50 $\mu$Ci/mmol) used for affinity labeling studies. The radioactive carbon was located in the carboxyl moiety adjacent to the benzoyl group in FSBA, not in the adenine, to ensure that the label remained attached to the peptide during isolation and analyses. Concentration of the radioactive FSBA was determined by UV absorbance as described above and correlated with the counts per minute of $^{14}$C, as determined by scintillation counting in Betafluor (National Diagnostics).

Monoclonal antibodies specific for SV40 T were gifts from E. Harlow (PAb419 and PAb423 [21]), E. Gurney (PAb101 [7]), and D. Lane (PAb204 [24]). Antibodies PAb419, PAb423, and PAb101 were purified from tissue culture supernatant fluids when necessary, using protein A-Sepharose. PAb204 was purified by immunoaffinity chromatography using goat anti-mouse immunoglobulin G (IgG) bound to Sepharose beads (Cooper Scientific). Aliquots of purified antibody were stored at −20°C.

**Purification of SV40 T antigen.** SV40 T antigen was obtained from either extracts of CV-1P monkey cells infected with *cs*1085, a mutant SV40 that overexpresses T (17), or from extracts of Sf9 insect cells infected with a recombinant baculovirus (vAc373T2) encoding the SV40 T gene (28). *cs*1085-encoded T was extracted 48 h postinfection, and vAc373T2-encoded T was extracted 42 h postinfection. For infection of Sf9 cells by vAc373T2 (multiplicity of infection, 10), the amount of T synthesis was measured several times after infection and was first detected at approximately 22 h and reached a plateau at approximately 40 h. There appeared to be more difference in the specific activity of two preparations of T from the same source than there was between that from the two different sources.

T protein was purified from infected cell extracts by immunoaffinity chromatography using triethylamine elution (34). Only eluted fractions containing T at less than pH 9.0 were then neutralized with 0.1 M Tris-$SO_4$ to pH 7 in 5% glycerol and used for these assays. The concentration of T was determined by UV spectroscopy with an optical density at $A_{280}$ of 1 = 12.3 $\mu$M, using aldolase as a control. If needed, T was centrifuged in a Centricon-30 (Amicon Corp.) to concentrate it and to change the buffer. By sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, T was no less than 95% of the protein present. Samples of FSBA-modified T containing bovine serum albumin were quantitated by enzyme-linked immunoadsorbent assay (ELISA) (22) performed with antigen bound to nitrocellulose in a dot-blot apparatus (Schleicher & Schuell) using $^{125}$I-labeled IgG specific for mouse IgG (Amersham). Known concentrations of T were titrated in parallel in the ELISA, and a pool of purified PAb419 and PAb101 (or PAb423) was used as the primary antibody. Assays were in duplicate, using three concentrations of the unknown sample, and the bound $^{125}$I-labeled IgG was quantitated by scintillation counting in Betaflour. If the samples contained [$^{14}$C]FSBA-bound T, they were counted on three different channels with windows of 0 to 400, 400 to 670, and 0 to 1,000. The 400 to 670 window detected ≥95% of the $^{14}$C and could be compared with [$^{14}$C]FSBA controls and subtracted from the total counts of $^{125}$I in the ELISA.

**Modification of T with FSBA.** The modification procedure was essentially that of Zoller and Taylor (39); T protein at 0.2 to 0.7 mg/ml was incubated at 30 to 37°C in a solution consisting of 50 mM Tris-$SO_4$ (pH 7), 1 mg of bovine serum albumin per ml, 2.5% glycerol, and millimolar FSBA dissolved in DMSO (making up 2% of the total volume at maximum). After incubation, the reaction was placed on ice and quenched with 10 mM dithiothreitol. Free nucleotides were removed by gel filtration in Bio-Gel-P10 (Bio-Rad Laboratories) in columns (0.5 by 3.6 cm) equilibrated in a solution consisting of 50 mM Tris hydrochloride (pH 7.8), 10% glycerol, 1 mM dithiothreitol, 0.1 M NaCl, and 0.05% Nonidet P-40. Fractions (100 $\mu$l) were collected, and aliquots were analyzed by nitrocellulose blot ELISA to detect the presence of T and by scintillation counting for the presence of [$^{14}$C]FSBA. Pools of T protein were made and reanalyzed by blot ELISA and for bound $^{14}$C in parallel with a standard [$^{14}$C]FSBA to assess the ratio of moles of FSBA-bound T to free T. Any remaining noncovalently bound label was removed from the protein by washing the nitrocellulose three times with 6 M guanidine-HCl (the protein-free [$^{14}$C]FSBA standard was not washed). One set of duplicate samples was then dried for direct scintillation counting, while the second set was analyzed by ELISA as described above. Pools of T were quick-frozen and stored at −70°C.

**Isolation of covalently modified peptide.** T (0.5 to 1 mg) was incubated with [$^{14}$C]FSBA as described above. To obtain efficient labeling of a majority of the protein for peptide analyses, two additions of 1.5 mM [$^{14}$C]FSBA were made during a 2-h incubation at 30°C. Under these conditions, the molar ratio of labeling FSBA to T was ≥0.95. $^{14}$C-labeled protein was isolated by SDS-polyacrylamide gel electrophoresis. The $^{14}$C-labeled T bands were excised from a 10% gel and eluted in a solution consisting of 50 mM $NH_4HCO_3$ (pH 8), 0.1% SDS, and 3% β-mercaptoethanol, followed by filtration through a membrane filter (45-$\mu$m pore size; Millipore Corp.) and by lyophilization.

For cyanogen bromide (CNBr) cleavage, lyophilized $^{14}$C-labeled protein was dissolved in 200 $\mu$l of degassed 75% formic acid containing a saturated solution of fresh CNBr (Sigma) and incubated in the dark with gentle rocking overnight at room temperature. A second addition of 200 $\mu$l of fresh CNBr was added, and the incubation was continued for another 24 h. After addition of 1 ml of $H_2O$, the sample was dried on a Speedy Vac. The material was then dissolved in 1 ml of $H_2O$ and dried three times. Before being analyzed

by electrophoresis in a 15 or 20% acrylamide gel, the material was dissolved in SDS-gel buffer containing bromphenol blue dye and neutralized with ammonium hydroxide. The markers, including carbonic anhydrase (26.6 kilodaltons [kDa]), myosin (12.5 kDa), and aprotinin (9 kDa), were run in parallel with the CNBr-treated protein at 100 V. After being stained and destained, the gel was treated with fluor to help detect the $^{14}C$ (Amplify; Amersham), then dried, and exposed to X-ray film.

For preliminary analyses of the location of the $^{14}C$-labeled residue in the protein, lyophilized material eluted from the gel was suspended in immunoprecipitation buffer (50 mM Tris hydrochloride [pH 8.5], 0.1 M NaCl, 0.5% Nonidet P-40) and incubated for 2 h at room temp with protein A-Sepharose and antibody. The epitope for PAb204 includes residues 453 to 469 (19), the epitope for PAb419 is located within residues 1 to 82, (21), the epitope for PAb101 is located within residues 512 to 699 (S. E. Mole and D. P. Lane, personal communication), and the epitope for PAb423 includes the C-terminal 8 residues (32). Each of the four monoclonal antibodies recognizes denatured protein. The complexes were washed with 50 mM $NH_4HCO_3$ (pH 8) buffer at 4°C. Buffer (100 μl) was added to paired samples of immunoprecipitated protein with or without 5 μg of trypsin (treated with tosylphenylalanine chloromethyl ketone [TPCK]), and the mixture was incubated at 4°C for 5 to 30 min. The immunoprecipitates were washed with 0.5 M Tris-LiCl (pH 8) and suspended in SDS-gel electrophoresis buffer for analysis in 15% gels. Alternately, partial tryptic digests were performed in SDS-polyacrylamide gels and Western blots (immunoblots) were prepared by the method of Schwyzer et al. (32), with specific monoclonal antibodies to T. Gel bands of $^{14}C$-labeled T were excised and loaded into wells of an SDS-polyacrylamide gel along with 0, 0.5, 2, or 10 μg of TPCK-trypsin. The gel was electrophoresed at 50 V for 7 min and then stopped for 30 min. Electrophoresis was then continued at 50 V until the prestained Coomassie blue-stained markers (Sigma) were well separated. The gel was transferred onto nitrocellulose (pore size, 0.22 μm) by electrophoresis for 2 h at 450 mA in Tris-glycine–methanol–0.05% SDS. The nitrocellulose was incubated with antibody overnight at room temperature, followed by incubation with second antibody conjugated with alkaline phosphatase (22). Immunoreactive bands were visualized by reaction with fast red. Overlapping immunoreactive and radioactive peptides were used to map the position of the $^{14}C$-label to a tryptic peptide.

For tryptic peptide analyses, the solution eluted from the 10% gel was lyophilized and dissolved in 0.1 ml fresh performic acid on ice for 2 h (1.9 ml of 98% formic acid and 0.1 ml of $H_2O_2$, incubated at room temperature for 1 h). TPCK-trypsin (50 μg) was added to the solution before relyophilization. It was then suspended in 200 μl of 50 mM $NH_4HCO_3$ (pH 8) with 50 μg of additional TPCK-trypsin and incubated for 4 h at 37°C. Another 50 μg of trypsin was added, and the incubation was continued overnight. Again the solution was lyophilized. The ester bond of FSBA between the sugar and the [$^{14}C$]carboxylbenzoylsulfonyl group is easily cleaved in basic solutions, leaving a net negative charge at the carboxyl terminus. Therefore, to ensure that the charge on the isolated peptide was homogeneous, the lyophilized protein was treated with 100 μl of 0.1 M NaOH for 4 h at room temperature (39). The protein solution was placed on ice and precipitated to remove salt by the addition of 2 volumes of cold acetone (15 min on ice, followed by centrifugation for 30 min at 4°C at 13,500 × *g*). The pellet was washed with cold acetone and suspended in 10 μl of electrophoresis buffer. Samples of both the supernatant fluid and the resuspended pellet were scintillation counted to determine the efficiency of recovery of $^{14}C$-labeled material.

**Two-dimensional thin layer mapping of the tryptic peptides.** High-voltage electrophoresis was carried out on cellulose thin layer chromatography plates (TLC plates [20 by 20 cm]; Kodak) in 1.2% pyridine, 1.2% acetic acid (pH 4.7) for 1 to 2 h at 500 V with cooling (LKB apparatus). After the plates were dried, they were chromatographed for 6 to 8 h either in hydrophobic buffer (butanol-acetic acid-$H_2O$-pyridine [68:14:25:40] [39]) or in hydrophilic buffer (isobutyric acid-pyridine-acetic acid-butanol-$H_2O$ [65:5:3:2:29]; F buffer [31]). After the TLC plates were dried, they were exposed to X-ray film.

**ATPase assays.** After gel filtration and quantitation of the T preincubated with or without nucleotide, aliquots were assayed for ATPase activity in 25-μl reaction volumes in buffer (Tris hydrochloride [pH 7.8], 10% glycerol, 1 mM dithiothreitol, 0.1 M NaCl, 0.05% Nonidet P-40) containing 7 mM $MgCl_2$ and 1 to 100 μM ATP, including 10 nM [$\gamma$-$^{32}P$]ATP for detection in the assay. Assays were incubated at 37°C for 30 and 60 min before 1-μl samples were spotted on polyethyleneimine-cellulose (Brinkmann). The plates were chromatographed in 0.75 M $NaH_2PO_4$ (pH 3.5) to separate the [$^{32}P$]ATP from the free $^{32}PO_4$. The counts per minute of $^{32}P$ spots on the plate were determined by cutting and scintillation counting the TLC plates or by direct detection and quantitation with Betascope (Betagen). The numbers of picomoles of free $^{32}PO_4$ released per reaction were calculated.

## RESULTS

**Conditions for labeling T with FSBA.** The ATP analog FSBA binds specifically to the Mg-phosphate region in ATP binding sites. As shown for several enzymes, the covalent interaction blocks catalytic activity. Under specific conditions, incubation of purified SV40 T protein with FSBA resulted in the inactivation of the ATPase activity (Table 1). As described by others (18, 39), we found that efficient covalent labeling with FSBA required neutral pH and low glycerol concentration. The addition of MgATP to FSBA in the incubation protected T from inactivation as an ATPase (see experiment B in Table 1 for an example), indicating specificity of the analog for the ATP-binding site.

Experiments in Table 1 were performed with incubation at 37°C to achieve efficient inactivation; however, there was significant loss of enzyme activity in the control sample incubated without nucleotide. Upon further investigation, incubation at 30°C allowed recovery of sufficient activity in the control for analyses, although the labeling was less efficient. T and FSBA were cross-linked at 30°C at a concentration of 1 mM FSBA as shown later, but approximately 2 mM appeared to be required for complete labeling and inactivation of the ATPase. This could not be accomplished consistently in one incubation as a result of the partial insolubility of FSBA at high concentrations as seen by others (23). Initially, the results of partial labeling of T with FSBA proved to be difficult to interpret (e.g., using 1 mM at 30°C), until direct analyses of the stoichiometry of the binding reaction were performed by using radioactive FSBA as described below. For mapping experiments, consistent labeling at a molar ratio of ≥0.9 mol of FSBA to 1 mol of T was achieved by the addition of 1.5 mM [$^{14}C$]FSBA and

TABLE 1. Conditions and specificity of FSBA inactivation of T-ATPase[a]

| Conditions for preincubation | % Activity |
|---|---|
| Experiment A | |
| Control | |
| 0.1 M Tris-$SO_4$ (pH 7.0) | 100 |
| 2.5% glycerol | |
| 1% DMSO | |
| 1 mM FSBA in 1% DMSO | 0 |
| 0.1 M Tris-$SO_4$ (pH 7.0) | |
| 2.5% glycerol | |
| 0.1 M Tris-$SO_4$ (pH 7.5) | 39 |
| 0.1 M Tris-$SO_4$ (pH 8.0) | 17 |
| 5% glycerol | 57 |
| 10% glycerol | 47 |
| 10 mM $MgCl_2$ | 47 |
| 100 mM $MgCl_2$ | 76 |
| Experiment B | |
| Control as above | 100 |
| 1 mM FSBA | 5 |
| 1 mM FSBA + 1 mM ATP–5 mM $MgCl_2$ | 130 |
| 1 mM ATP–5 mM $MgCl_2$ | 172 |

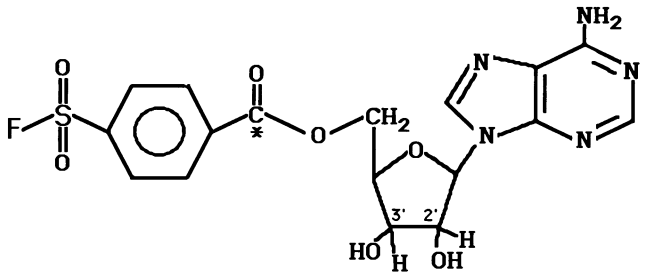
[a] Purified T protein (0.2 mg/ml) was incubated at 37°C for 60 min in the pH 7 Tris-$SO_4$ buffer described with or without modifications or additions of nucleotide. At the end of the incubation, dithiothreitol was added to 10 mM and the samples were gel filtered at 4°C in a solution consisting of 50 mM Tris hydrochloride (pH 7.8), 0.1 M NaCl, 0.05% Nonidet P-40, 1 mM dithiothreitol, and 10% glycerol. Fractions containing T were determined by $^{125}I$ ELISA and assayed for ATPase activity at 1 μM ATP for 30 min at 37°C, as described in Materials and Methods. The ATPase activity recovered in the controls was substantially reduced after initial incubation at 37°C; therefore, all following experiments were preincubated at 30°C (39).

incubation at 30°C for 1 h, followed by a second addition of 1.5 mM analog and incubation for another hour (39). We examined the specificity of the FSBA modification for the ATP-binding site further by analyses of $^{14}C$-labeled peptides.

These studies were performed successfully with purified T derived from either SV40 infection of monkey cells using the mutant virus *cs*1085 (17), or SV40-recombinant baculovirus infection of insect cells (28), both prepared as described in Materials and Methods. Purified T from infected insect cells was used for most of the experiments described below.

**FSBA bound specifically to one site in T.** We have predicted the location and tertiary structure of one ATP-binding site in SV40 T that includes a minimum of 110 residues from 418 to 528 (6). From this prediction, the residues most likely to interact with the Mg-phosphate of ATP were Glu-473 and Asp-474. In the ATP analog FSBA (Fig. 1), a carboxylbenzoylsulfonyl group substitutes for the Mg-phosphate in the ATP and cross-links to lysine or tyrosine residues in the protein. Lysine 476 of T is close to the predicted site of MgATP binding in T and was thought to be the linkage site for FSBA. A schematic diagram of the predicted structure of the ATP-binding site in T and the contracts of ATP with the protein are described in Bradley et al. (6).

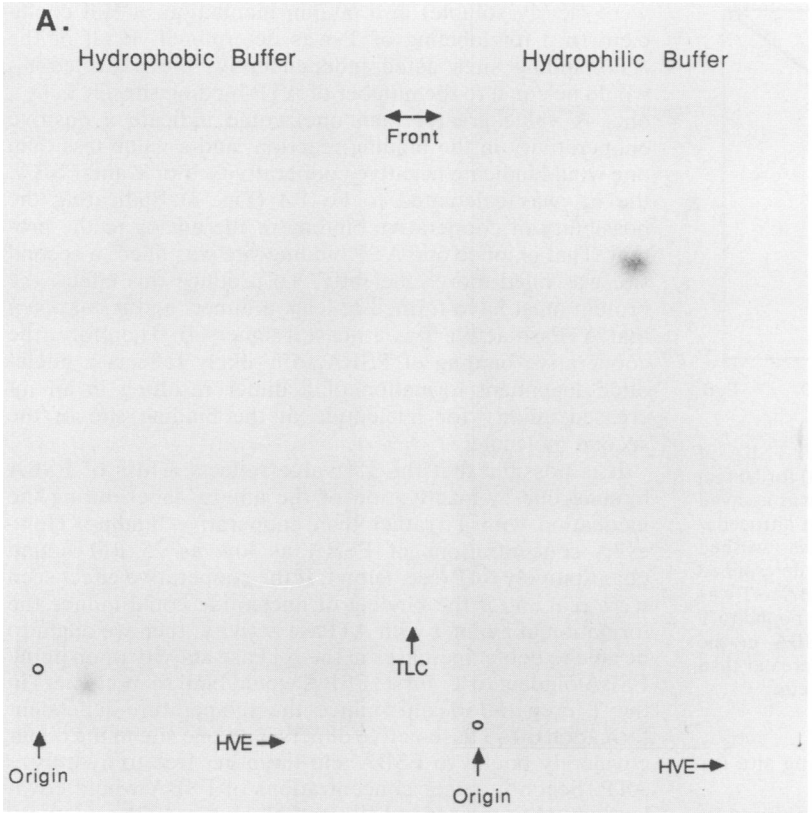
Labeling of T with [$^{14}C$]FSBA followed by exhaustive tryptic digestion produced a single, radioactively labeled peptide. $^{14}C$-labeled T was treated with base to hydrolyze the ester bond connecting the sugar and base of the nucleotide to the $^{14}C$-labeled carboxylbenzoylsulfonyl group (39), leaving a homogeneous $^{14}C$-labeled peptide with an additional carbonyl group and a net negative charge. The radioactively labeled tryptic peptide was isolated by two-dimensional techniques. In the first dimension, high-voltage electrophoresis moved the peptide slowly toward the cathode, indicating that the peptide was large and negatively charged. The use of two different buffers for the TLC system in the second dimension confirmed that the peptide was unique and strongly hydrophilic (Fig. 2A). No other radioactively labeled peptides were seen in five experiments, even after 4-months exposure of the TLC plates that had shown single radioactively labeled spots after less than 4-days exposure to X-ray film. Therefore, FSBA bound to one site in T.

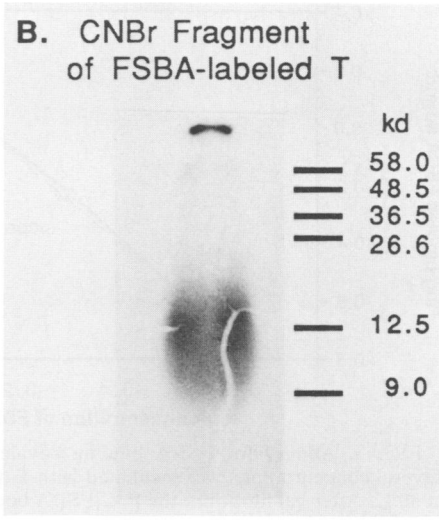


p-fluorosulfonylbenzoyl 5'-adenosine

FIG. 1. Covalent affinity analog FSBA and the location of the $^{14}C$-label as synthesized by Amersham (*). The electrophilic sulfonyl fluoride usually reacts with lysyl or tyrosyl residues in the ATP-binding sites of the protein (for a review, see reference 12). By exposing the complexed peptide to a mild base (0.1 M NaOH at room temperature), the ester bond between the sugar and the carboxylbenzoylsulfonyl group is cleaved, leaving a net negatively charge on the [$^{14}C$]carbonyl moiety on the protein. Treatment with base was used in mapping experiments to produce a uniquely labeled peptide for isolation and analysis.

The location of the FSBA-labeled residue in the proposed ATP-binding site was determined by cleavage of FSBA-labeled T with CNBr and by partial tryptic analyses paired with T-specific monoclonal antibody recognition. First, the ATP-binding site in SV40 T was found to be located in the largest CNBr fragment of the protein between methionines 412 and 528 (6, 10). Exhaustive digestion of [$^{14}C$]FSBA-labeled T with CNBr repeatedly produced a large band migrating at approximately 12-kDa in acrylamide gels (see example in Fig. 2B). No smaller peptides were generated. Therefore, the FSBA bound to one region in T that coincided with the previously mapped covalent affinity binding sites for oADP (10) and MgAMP (6). Further support for the prediction of the $^{14}C$-labeled residue in T was accomplished by using T-specific monoclonal antibodies. Partial trypsinization of immunoprecipitates left 26- to 36-kDa $^{14}C$-labeled peptides associated with monoclonal antibodies PAb204 and PAb101 (data not shown). In addition, partial trypsinization followed by Western blot analyses was used for mapping by the method of Schwyzer et al. (32). A distinct pattern of $^{14}C$-labeled, partial tryptic peptides derived from [$^{14}C$]FSBA-labeled T was seen with a predominant 36-kDa band (Fig. 2C, panel a). These tryptic digestions were immunoblotted by using several T-specific monoclonal antibodies, including PAb204 and PAb101. PAb204 recognized radioactive peptides of ≥42 kDa known to extend from near residue 131, but none of ~36 kDa (only a mixed blot is shown in panel b). Whereas PAb101, which binds a more C-terminal region, readily detected 31- to 36-kDa radioactive peptides (Fig. 2C, panels b and c). Therefore, $^{14}C$-labeled 31- to 36-kDa fragments are from the C terminus and most likely



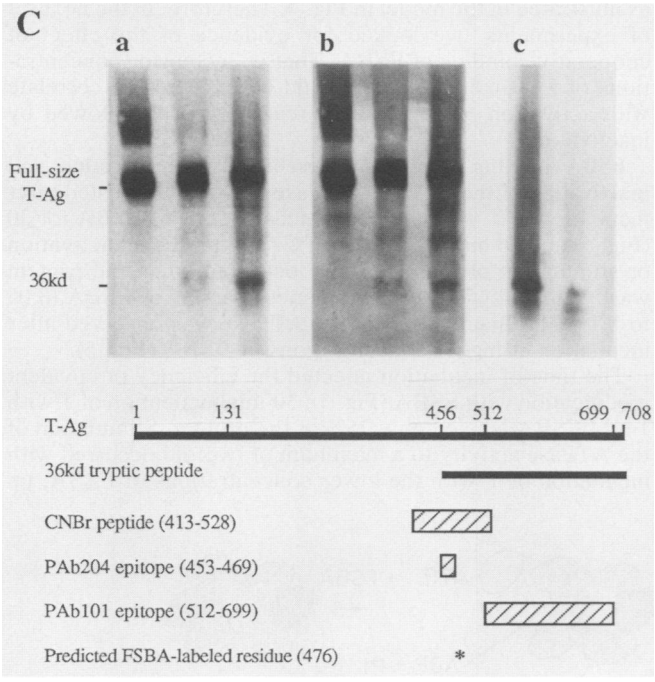




FIG. 2. Tryptic and CNBr peptide mapping of the [$^{14}$C]FSBA-labeled T protein. After T was labeled with [$^{14}$C]FSBA for 2 h, the $^{14}$C-labeled T was isolated by electrophoresis in 10% SDS–polyacrylamide gels and treated with TPCK-trypsin or CNBr as described in Materials and Methods. (A) $^{14}$C-labeled tryptic peptides were spotted onto cellulose TLC plates and subjected to high-voltage electrophoresis (HVE) in the first dimension. After the TLC plates were dried, they were developed in the second dimension in one of two buffer systems, hydrophobic buffer (39) on the left or hydrophilic buffer (F buffer [31]) on the right. After the plates were dried, they were exposed to X-ray film for 4 days (shown) and 4 months. (B) One experiment of three is shown in which $^{14}$C-labeled T was cleaved with CNBr and analyzed by SDS-acrylamide gel electrophoresis. Marker proteins run in parallel included myosin (12.5 kDa) and aprotinin (9 kDa) in a 15% acrylamide separating gel. (C) An example of the mapping technique of Schwyzer et al. (32) used for identifying the FSBA-modified tryptic fragment. Panel a shows a partial tryptic digest of the [$^{14}$C]FSBA-labeled T. On the right are two immunoblots of the partial tryptic digest. Panel b shows a blot probed with both the T-specific monoclonal antibodies PAb204 and PAb101. Panel c shows part of a parallel blot probed with PAb101 alone. Below the data is a diagram of the location of the partial tryptic peptides derived from these immunoblots (and from others not shown), the locations of the large CNBr fragment, and the epitopes of the monoclonal antibodies PAb204 and PAb101. Prominent $^{14}$C-labeled and PAb101-reactive 31- to 36-kDa fragments were detected in several different experiments. There was no recognition of these 31- to 36-kDa peptides by PAb204, but larger $^{14}$C-labeled fragments of ~42 and 58 to 68 kDa extending from the frequent cleavage site near residue 131 were detected (32). The $^{14}$C-labeled 31- to 36-kDa peptides most likely extend from an N-terminal cleavage at Arg-456 in the middle of the PAb204 epitope to the C-terminal residue 699 or 708. T-Ag, T antigen; Kd, kDa.

arose from tryptic digestion in the PAb204 epitope at Arg-456. Since the $^{14}$C label appeared in the CNBr fragment and either the PAb204-reactive 42-kDa peptide or the PAb101-reactive 36-kDa peptide, the labeled residue would have to be just C terminal to the PAb204 epitope (Fig. 2C). Any smaller fragments having both immunoreactivity and radioactive label may have arisen out of coincidence. These data

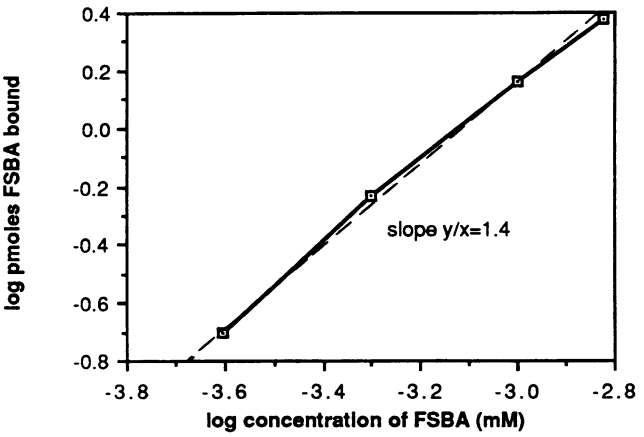



FIG. 3. Affinity for FSBA binding covalently to T. FSBA at several concentrations was incubated with T (0.5 mg/ml) for 60 min at 30°C. After gel filtration, the [$^{14}$C]FSBA bound to T was assayed at 2 volumes in duplicate by trapping the complexes on nitrocellulose. Before scintillation counting, the nitrocellulose was washed three times with 6 M guanidine-HCl (18). T content was determined in parallel by ELISA. The Hill coefficient for binding FSBA to T was calculated as the slope of the log plot of moles of FSBA bound to T ($y$ axis) versus the log of the concentration of FSBA in the incubation ($x$ axis). Values for the slope of the line ($y/x$) greater than 1.0 indicated cooperative binding of the reagent to protein.

are wholly consistent with our predicted combining site for FSBA at Lys-476.

**Determination of the affinity of FSBA for T.** Analyses of several labeling experiments indicated that FSBA labeled T at a molar ratio of one. Using a 60-min labeling time, the concentration of FSBA added to T was titrated to determine the binding affinity. Although saturation binding with FSBA was unreliable as a result of insolubility of high concentrations of FSBA in the reaction mixture (≥2 mM), an estimated binding constant ($S_{0.5}$) for FSBA to T was 1 mM (for example, see 50% binding of T at 1 mM FSBA [Fig. 6C]). This is significantly lower than the affinity of T for ATP (0.5 μM; P. Clertant and M. Bradley, unpublished observations), but the difference was similar to that found for FSBA versus ATP binding to RecA (23). In a 30-min incubation, the covalent labeling was less efficient, with an estimated $S_{0.5}$ of 2 mM, with 4 mM FSBA required for complete complex formation. No concentration of the analog or T protein tested (or extended incubation time) resulted in labeling at a molar ratio of more than one. Again, this indicated that there was only one site for FSBA binding in the protein.

Using concentrations of 0.25 to 1.5 mM FSBA (which were clearly soluble) in a 60-min incubation, a Hill coefficient ($n_H$) for labeling of T was determined. If all of the ATP-binding sites acted independently, the expected $n_H$ would be equal to the number of ATP-binding sites in T, i.e., one. A value greater than one would indicate a positive cooperativity in the binding reaction, and a value less than one would indicate negative cooperativity. For T and FSBA, the $n_H$ was calculated to be 1.4 (Fig. 3), indicating the possibility of cooperative binding of the analog to the protein. That is, once one ATP binding site was filled, a second site was filled more efficiently. To produce this effect, the protein must have formed at least a dimer, and it is known that ATPase-active T is at least a dimer (4). Therefore, the cooperative binding of FSBA to T likely reflects a nucleotide-dependent formation of a dimer resulting in an increased affinity for nucleotide in the binding site of the second molecule.

It is possible that the 1.4 value reflects a loss of FSBA binding due to inactivation of the affinity label during the incubation with T, rather than cooperative binding. However, concentrations of FSBA as low as 25 μM bound quantitatively to T (see below). If the cooperative effect seen were real and if the binding of nucleotide could induce the formation of a dimer with ATPase activity, then we ought to be able to detect increases in the ATPase activity upon initial FSBA binding to T. First, FSBA would bind to monomers in the T preparation and induce the temperature-dependent formation of ATPase-active dimers with one site in the dimer covalently bound to FSBA and the other free to hydrolyze ATP. Second, higher concentrations of FSBA would covalently bind to the second site and block the ATPase activity as illustrated in the model in Fig. 4. Therefore, in the next set of experiments, we looked for evidence of the effect of cooperative binding of FSBA. That is, increasing concentrations of FSBA bound to T would be expected to correlate with activation of the ATPase reactivity of T followed by inactivation.

**FSBA binding to T was correlated with activation and inactivation of the ATPase.** ATPase activity was tested after incubation of T with various concentrations of FSBA for 30 (Fig. 5) and 60 min (Fig. 6) at 30°C. The predicted activation or stimulation of the T-ATPase occurred when the protein was incubated with the lower concentrations of FSBA (0.05 to <1 mM). Inactivation of the ATPase was observed after incubation at higher concentrations of FSBA (Fig. 6).

The time of incubation affected the efficiency of covalent modification with FSBA (Fig. 5); 30-min incubation of T with 1 mM FSBA labeled only 25% of the protein. Stimulation of the ATPase activity to a maximum of twofold occurred with incubation of T with the lower concentrations of FSBA, up

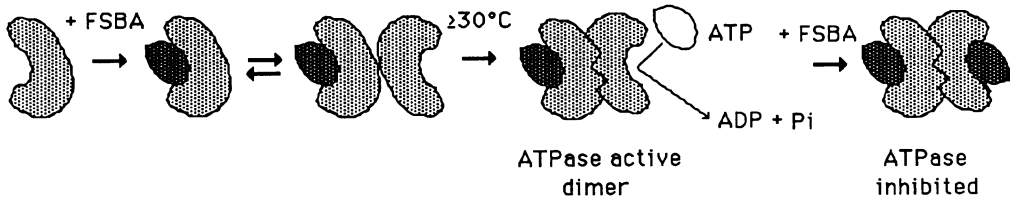



FIG. 4. Model for the interaction of adenine nucleotide with T. It is proposed that binding of ATP, AMPPCP, or FSBA to T induces a conformational change in the protein. With this change, a nucleotide-bound monomer is predicted to form a dimer that binds nucleotide with a higher affinity to the second site. This would be consistent with the cooperative binding reaction shown in Fig. 3. Even with FSBA bound covalently to one site, the second site could catalyze the hydrolysis of ATP to ADP and $P_i$. When a covalent adduct is formed with FSBA in both sites, the ATPase would then be inhibited. This figure was adapted from a depiction of cooperative binding shown in Darnell et al. (13).

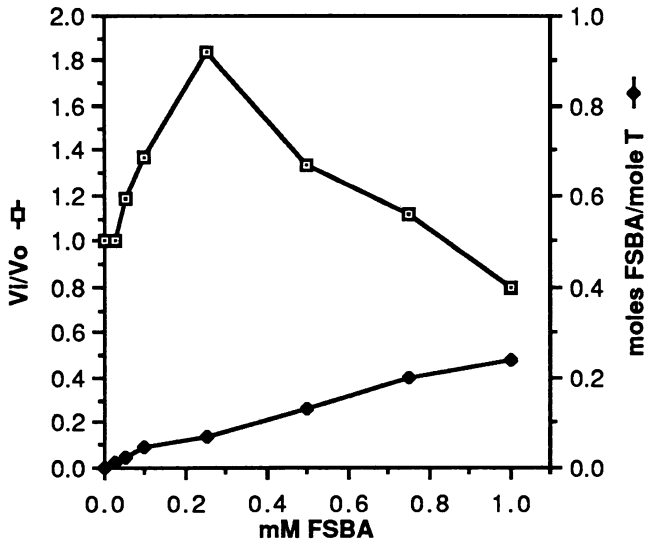



FIG. 5. Activation of T-ATPase by binding low concentrations of FSBA. Concentrations of [$^{14}$C]FSBA from 0.025 to 1.0 mM were incubated with T (0.5 mg/ml) for 30 min at 30°C. After gel filtration, the ATPase activity (Vi/Vo) and the moles of [$^{14}$C]FSBA bound per mole of T were assayed as described in Materials and Methods. The ATPase activity was plotted compared with that of the control incubated without added nucleotide (Vo). In this and other experiments, when the incubation was 30 min or less, 1 mM FSBA labeled T at a molar ratio of ~0.25 or 25%.

to 0.25 mM (Vi/Vo = 1.83 in Fig. 5). At concentrations greater than 0.25 mM, there was less apparent stimulation. Notice that the level of ATPase activity recovered after treatment with 1 mM FSBA was similar to that of the nucleotide-free control. These results lead one to suspect that as some T was activated by FSBA, it could then be inactivated by binding additional analog.

With more efficient covalent labeling of T in a 60-min incubation, T-ATPase was still stimulated by 0.25 mM FSBA but was inactivated by concentrations greater than 0.5 mM to below the activity of the nucleotide-free control. As a control for this experiment, a sample of T was incubated with another analog of ATP, AMPPCP. AMPPCP at concentrations of 1 to 1.5 mM induced maximal stimulation of the ATPase activity (up to twofold) without covalent association of the nucleotide. In the experiment shown in Fig. 6A, AMPPCP-treated T had 80% more activity than the ATPase activity of the nucleotide-free control, and T pretreated with 1.5 mM FSBA had 35% less activity than the control. The data plotted in Fig. 6B show AMPPCP-treated T as the most active for ATPase activity (Vi/Vo = 1.8) and then show that an increase in covalent modification of T by FSBA is directly proportional to a decrease in ATPase activity. If T molecules were labeled with FSBA at a molar ratio of one (see the right-hand $y$ axis of Fig. 6B), the enzyme activity would have been completely inactivated. Note that ~50% FSBA labeling resulted in ~50% recovery of the potential ATPase activity (Vi/Vo = 1). With these findings, it appeared that FSBA both activated and inactivated the enzyme by binding to the protein. Since only one site was identified, the stimulatory activity of nucleotide had to have occurred at the catalytic site in T.

Stimulation of the ATPase also occurred if the protein was incubated with millimolar concentrations of ATP (data not shown). This effect was shown in Table 1 (experiment B), when addition of ATP to the preincubation stimulated the ATPase activity of T compared with that of the control. However, it is important to note that the ATPase was stimulated by the nonhydrolyzable analogs FSBA and AMPPCP, therefore no hydrolysis of ATP was required for the activation. The reaction did require elevated temperature, since performing the incubation with nucleotide at room temperature or 4°C gave little or no response (data not shown), while incubation at 30 or 37°C did. For FSBA, the percent activation was shown to be dependent upon the concentration of analog in the reaction that bound to T.

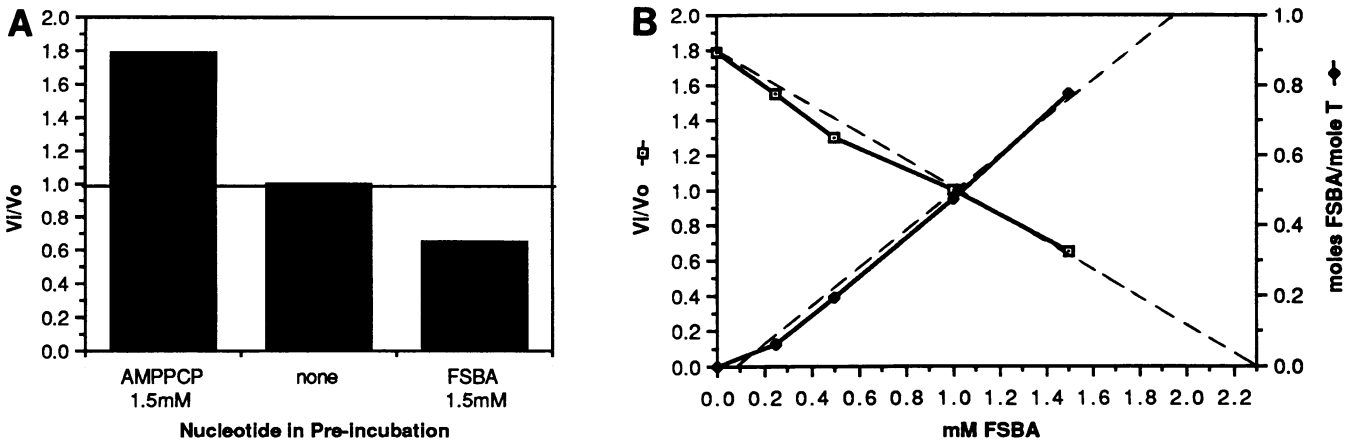



FIG. 6. Activation and inhibition of the T-ATPase by FSBA correlate with the covalent binding. T (0.5 mg/ml) was incubated with 0.25 to 1.5 mM FSBA for 60 min at 30°C and assayed as for Fig. 5. (A) The effects of incubation with 1.5 mM FSBA on the ATPase activity were compared directly with incubation of T without nucleotide and with 1.5 mM AMPPCP. (B) Results of titrating FSBA with T were plotted as the molar ratio of FSBA bound to T (moles of FSBA per mole of T on the right $y$ axis) versus the concentration of FSBA in the incubation reaction. On the left $y$ axis is the ratio of the ATPase activity of the FSBA-treated samples compared with the activity of the nucleotide-free control (Vi/Vo) with the maximum activity as that of T incubated with 1.5 mM AMPPCP as seen in Fig. 6a. FSBA at 1 mM labeled T at a molar ratio of ~50%. According to these data (the lines plotted by least-squares analyses), the concentration of FSBA estimated for 100% inhibition of ATPase was 2.3 mM; for 100% labeling, it was 2 mM. However, concentrations of ≥2 mM were not consistently soluble in the incubation mixture (23).

## DISCUSSION

The covalent affinity analog for ATP FSBA cross-linked specifically to a unique tryptic peptide of SV40 large T. The peptide mapped to within the predicted ATP-binding site of the protein between residues 418 to 528 (6). The covalent association of FSBA with T occurred at a molar ratio of approximately 1:1, with ≥95% of T labeled. ATPase activity was inhibited when most of the ATP sites were filled with the sulfonylbenzoyl 5′-adenosine analog, but surprisingly, when less than 50% of the T was complexed, the ATPase activity was stimulated. This means that only one site in T was associated with both activation and inactivation of the ATPase. An explanation of the phenomenon appears to be that binding of nucleotide induces the formation of an ATPase active dimer, resulting in one site bound to FSBA and one site free for catalysis. The finding that FSBA binds cooperatively to T supports the hypothesis that the ATP- binding site has two roles (Fig. 4).

Nucleotide-binding sites in several enzymes from *E. coli*, animal cells, and viruses, are used for both hydrolysis and regulation of the active state of the protein. Proteins with known structures have been used to model the ATP-binding site in SV40 T, including the ATP-binding enzyme adenylate kinase, the GTP-binding translation elongation factor EF-Tu, and the GTP-binding oncogene product Ras (6). There are other proteins that have been extensively investigated biochemically that can be classified in the same functional group (T. A. Webster and M. K. Bradley, unpublished observations) such as RecA (27) and DnaA (33). In each case, it has been shown that the nucleotide binding to the protein served to regulate the active state of the enzyme by changing the conformation, usually inducing at least a dimeric form. RecA, for example, showed cooperative binding to nucleotide associated with oligomerization, resulting in increased ATPase activity. Therefore, these data on T not only support the tertiary model for the ATP-binding site presented, they indicate that this structure in T may be part of a class of sites associated with enzyme regulation.

It is interesting to compare these results using FSBA to those using the covalent affinity analog oATP. Both analogs inhibited the ATPase activity of T and bound to the same CNBr peptide. An important difference was that oATP labeled T to a maximum of only ~50% (9), while FSBA labeled >90% of T. Cross-linking of oATP between its modified ribose moiety and the protein required hydrolysis to oADP (10), implying that there was a change in the nucleotide-binding site after hydrolysis of ATP to ADP. In contrast, T covalently bound almost all of the nonhydrolyzable analog FSBA to the catalytic site (in the Mg-phosphate pocket). However, when less than 50% of T was labeled with FSBA, ATPase activity was stimulated, rather than inhibited, with inhibition occurring after 50 to 100% of T was bound to FSBA. Apparently there is a subset or form of T that binds covalently to FSBA that does not bind oADP.

What is this other subset of nucleotide-bound T? One possibility is that it is involved in initiating the helicase activity shown to be associated with T by Stahl et al. (35). We and others have found that, although helicase requires the ATPase activity, it is apparently a special subset of T. Most of the ATPase-active T did not comigrate with helicase-active fractions upon zonal sedimentation (14, 35; B. Weiner and M. K. Bradley, unpublished observations). It is also known that the helicase activity requires millimolar concentrations of ATP, while T-ATPase requires micromolar ATP ($K_m$ = 5 to 50 μM; [20; Bradley and Clertant, unpublished observations]). In accordance with our model (Fig. 4), helicase activity might be constituted by a monomer of ATP-bound T binding forked DNA. A second molecule of T would dimerize with it to form the ATPase-active T able to melt the double-stranded DNA. Consistent with this proposal and the above data, it has been reported that T-ATPase was stimulated by single-stranded DNA, but only if millimolar concentrations of ATP were used as substrate (20, 37). According to this model, if T were induced to form an ATPase-active dimer in solution without DNA, there would be no helicase activity. Consistent with this hypothesis, we and others have found that helicase activity migrated at less than 7S (14, 35; Weiner and Bradley, unpublished observations). Studies are in progress using the FSBA analog to analyze the relationship between helicase and ATPase-active T.

Apparently, ATP has another role in viral DNA replication, since it has been reported that millimolar concentrations of ATP affected the ability of T to bind specifically to viral DNA (2, 15, 38). Specifically, ATP was required for T to open the SV40 origin of replication similar to the action of DnaA on the *E. coli* origin of replication (3, 29, 33). For SV40 T, hydrolysis of the ATP for helicase or ATPase activity appeared to be necessary only after the origin DNA was opened, perhaps to form a replication fork. With these steps in mind, mutations within the ATP-binding site of T could produce regulatory as well as catalytic defects leading to inactivation of the replication function. Therefore, we propose that ATP-mediated regulation of T activity is likely to be of primary importance in T-dependent SV40 DNA replication activity.

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