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ATP-Reactive Sites in the Bacteriophage λ Packaging Protein Terminase Lie in the N-Termini of Its Subunits, gpA and gpNu1

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ATP-reactive sites in terminase and its subunits have been successfully identified using three different affinity analogs of ATP (2- and 8-azidoATP and FITC). GpA, the larger subunit of terminase, was shown to have a higher affinity for these analogs than gpNu1, the smaller subunit. The suitability of these reagents as affinity analogs of ATP was demonstrated by ATP protection experiments and *in vitro* assays done with the modified proteins. These analogs were thus shown to modify the ATP-reactive sites. The results obtained from these experiments also indicate the importance of subunit–subunit interactions in the holoenzyme. Terminase, gpA, and gpNu1 were modified with these analogs and the ATP-reactive sites were identified by isolating the modified peptide by reverse-phase chromatography. The sequence analysis of the modified peptides indicates a region including amino acids 18–35 in the N-terminus of gpNu1 and a region including amino acids 59–85 in the N-terminus of gpA as being the ATP-reactive sites.

INTRODUCTION

An essential component of the λ packaging machinery is the phage-encoded enzyme terminase (reviewed by Becker and Murialdo (1990); Murialdo (1991), and Catalano *et al.* (1995). Terminase is a multifunctional enzyme. It is made of one subunit of gpA (M_r 72,280) and two subunits of gpNu1 (M_r 20,444). Terminase recognizes specific nucleotide sequences on concatameric λ DNA called *cos,* for cohesive end site. *cos* is composed of three subsites, *cosQ, cosN,* and *cosB.* Recognition for the *cos* site is conferred by the binding of gpNu1 to three sites, R1, R2, and R3, within *cosB* (Shinder *et al.,* 1988). Terminase then introduces staggered nicks at *cosN* to generate the 12-bp single-stranded overhangs of mature λ chromosome. Different activities associated with the holoenzyme include site-specific DNA binding, helicase activity, site-specific DNA nicking, ATP binding and hydrolysis, prohead binding, and translocase activity. Most of these activities have been well characterized *in vitro.* Purification and characterization of gpA (Parris *et al.,* 1994) has revealed that gpA can account for nearly all the *in vitro* ATPase (Rubinchik *et al.,* 1994b), helicase (Rubinchik *et al.,* 1994b; Yang and Catalano, 1997), *cos* cleavage (Rubinchik *et al.,* 1994a), and translocase activity of terminase (Rubinchik *et al.,* 1995). However, neither gpA nor gpNu1 is capable of promoting packaging on its own.

ATP is a crucial cofactor for terminase action. The holoenzyme and each individual subunit interacts with ATP in some manner. ATP and *cosB* are both important in determining the efficiency and accuracy of *cos* cleavage (Higgins *et al.,* 1988; Cue and Feiss, 1993a,b; Higgins and Becker, 1994). Strand separation of the 12-bp overhangs formed after *cosN* cleavage (Higgins *et al.,* 1988) and translocation of DNA into the proheads requires ATP hydrolysis. Gold and Becker (1983) showed that terminase has a DNA-dependent ATPase activity *in vitro* with ADP and P_i as the products. Becker and Gold (1988) examined the amino acid sequence of gpNu1 and compared it to several ATPases. They predicted that gpNu1 would be an ATP-reactive protein because it contained the canonical Walker "A" and "B" sites common to many ATPases and GTPases (Walker *et al.,* 1982; Saraste *et al.,* 1990). A Walker A motif or a "P-loop" is a glycine-rich segment followed by a lysine. GpNu1 has such a putative P-loop between residues 29 and 36 and gpA has a P-loop between residues 491 and 498. However, a change from the highly conserved lysine 35 in the A site to glutamic acid yielded a mutant terminase that showed ATPase activity similar to that of the wild type but had reduced packaging activity (Yang, 1993). The mutant gpNu1 was shown to have very weak DNA binding activity (Yang, 1993). Similar observations have also been reported by Hwang and Feiss (1997). They showed that the K35A and K35E mutant terminases had altered affinity and specificity of interactions with the DNA. This suggests that the mutation at K35 affects the DNA binding activity of terminase. Similar mutations that changed the conserved lysine K497 in the putative P-loop of gpA were found to be lethal (Hwang and Feiss, 1996). Phages carrying

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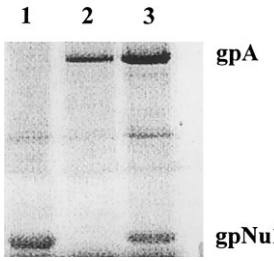


FIG. 1. Covalent modification by FITC. GpNu1 (lane 1), gpA (lane 2), and terminase (lane 3) were modified with FITC (0.5 mM) as described under Materials and Methods and electrophoresed on an SDS–polyacrylamide gel. The gel was scanned using the blue fluorescence scanner on a STORM (Molecular Dynamics) to visualize the covalent incorporation of FITC.

these mutations were defective in *cos* cleavage *in vivo.* However, *in vitro* packaging studies done with these mutant terminases showed packaging activity similar to that of wild type. Thus, none of the mutations completely eliminate ATPase or ATPase-related packaging activity, which suggests that the sequences thought to be the putative ATP binding sites may not be the actual ATP-reactive sites.

Kinetic analysis done by Tomka and Catalano (1993) showed that terminase has two ATP binding sites, one with high affinity and K_m of 5 μM and a second low-affinity site with a K_m of 13 mM. GpA has also shown to have an *in vitro* DNA-dependent ATPase activity, while gpNu1 was shown to have a DNA-independent ATPase activity (Parris *et al.,* 1988; Rubinchik *et al.,* 1994b). Photoaffinity labeling studies using 8-azidoATP have confirmed the presence of a high-affinity ATP center in gpA and a low-affinity ATP center in gpNu1 (Higgins and Becker, 1995; Hwang *et al.,* 1996). Similar experiments using 8-azidoATP have been carried out by Hamada *et al.*(1987) on the terminase of T3 phage.

Since there now appears to be a multiplicity of ATP-reactive motifs in proteins, we have begun a detailed analysis to identify the residues in terminase and its subunits, which directly interact with the different portions of the ATP molecule. In this report we show that there are specific domains which can interact with the purine nucleotide moiety of the triphosphate.

RESULTS

Modification by FITC

Terminase, gpA, and gpNu1 were modified by FITC and analyzed by SDS–polyacrylamide gel electrophoresis. The modified proteins were visualized by scanning the gel on a STORM (Molecular Dynamics) using the blue fluorescence scanner. As seen in Fig. 1, while both gpA and gpNu1 show incorporation of FITC, gpA showed four times more incorporation than gpNu1. An FITC binding contaminant seen between gpA and gpNu1 is an artifact, as Coomasie blue staining of the same gel did not reveal any protein band corresponding to this position. Labeling with FITC, on a weight basis, was found to be at least 30% more efficient when it was done at 37°C instead of room temperature. However, analysis of various enzymatic activities showed that gpA was denatured upon prolonged incubations at 37°C, while temperature and time activity profiles of terminase and gpNu1 were more stable. Therefore, for analyzing the effect of covalent modification on the *in vitro* packaging and ATPase activities of terminase, gpA, and gpNu1, labeling with FITC was carried out at room temperature for 30 min and preparative labeling for isolation of labeled peptides was carried out at 37°C for 45 min.

Effect of FITC modification on the enzymatic activities

In order to confirm that the modification with FITC was specific for the ATP-reactive site(s), the modified proteins were assayed for their different ATP-dependent enzymatic activities. Incubation of terminase with increasing concentrations of FITC inactivated its *in vitro* packaging activity (Fig. 2A). Complete inhibition of the packaging activity of terminase was observed at 500 μM FITC. Covalent modification of gpNu1 under these conditions did not affect its packaging activity. However, partial

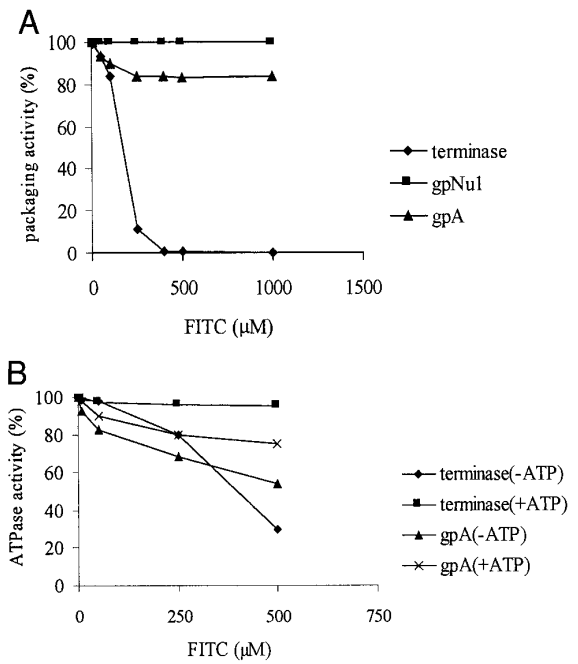


FIG. 2. (A) Inhibition of packaging activity of terminase, gpA, and gpNu1 by FITC. Terminase (1.5 μM), gpA (2.5 μM), and gpNu1 (10 μM) were incubated with the indicated concentrations of FITC for 30 min in the dark at room temperature and then assayed for packaging activity. (B) Inactivation of ATPase activity of terminase and gpA by FITC. Terminase (5 μM) and gpA (4 μM) were incubated in a total volume of 25 μl, in the dark at room temperature, for 30 min with different concentrations of FITC and then assayed for residual ATPase activity as described under Materials and Methods.

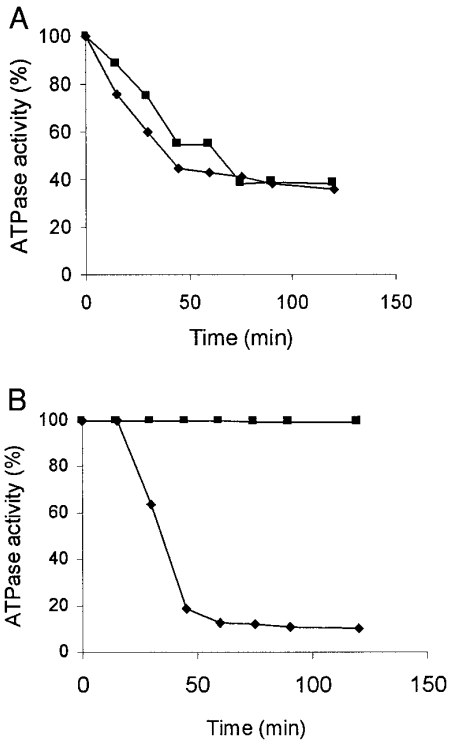


FIG. 3. (A) Time course of inactivation of the ATPase activity of gpA by FITC. GpA (30 μg) was incubated in a total volume of 100 μl (◆) in the presence or (■) in the absence of 500 μM FITC, in the dark at room temperature. At different times aliquots were removed and assayed for ATPase activity as described under Materials and Methods. (B) Time course of inactivation of the ATPase activity of terminase by FITC. Terminase (40 μg) was incubated in a total volume of 100 μl, (◆) in the presence or (■) in the absence of 500 μM FITC, in the dark at room temperature. At different times aliquots were removed and assayed for ATPase activity as described under Materials and Methods.

inactivation was observed at prolonged incubations. GpA lost 20% of its packaging activity at about 500 μM and any further increase in FITC concentration did not result in more inactivation. A time course of inactivation of *in vitro* ATPase activity of terminase and gpA is illustrated in Fig. 3. The presence of ATP was found to completely block the inactivation of ATPase activity of terminase by FITC (Fig. 2B). However, only partial protection by ATP was observed for gpA. This suggests that while there is specific labeling of essential residues in terminase holoenzyme by FITC, in gpA there may be some nonspecific labeling of nonessential residues.

Photoaffinity labeling of proheads, terminase, gpA, and gpNu1

The binding and photocrosslinking of 2- and 8-azidoATP is demonstrated in Figs. 4 and 5. GpA was found to have a higher affinity for 2- and 8-azidoATP than gpNu1. The 2- and 8-azidoATP also label a capsid protein, gpE. This is interesting in view of the fact that no ATPase activity has been found associated with purified proheads (unpublished results). As judged by phosphorimager scans of the dried gels, labeling with [α^{-32}P]8-N_3ATP and [β,γ^{-32}P]2-N_3ATP of gpA was seven- and fivefold greater, respectively, than that of gpNu1 (based on the subunit ratio of two molecules of gpNu1 to one molecule of gpA in a terminase protomer). Labeling of the gpA and gpNu1 was much stronger when photolabeled as a holoenzyme than when labeled as purified subunits. Thus, gpA and gpNu1 appear to interact more strongly with ATP when present as a holoenzyme than when separated.

To optimize the conditions for photolabeling, the effects of time of photolysis were examined. Maximum photoinsertion of either probe into gpA and gpNu1 was observed after approximately 2 min of UV irradiation. No labeling was observed when the samples were incubated with the photoprobe in the absence of UV light.

To show that 2- and 8-azidoATP are indeed substrate analogs and interact with the ATP binding site, the ability of terminase to hydrolyze [β,γ^{-32}P]2-N_3ATP was tested. Figure 6 shows the result of an experiment in which the rate of hydrolysis of [β,γ^{-32}P]2-N_3ATP by terminase and gpA was determined. The rate of hydrolysis of

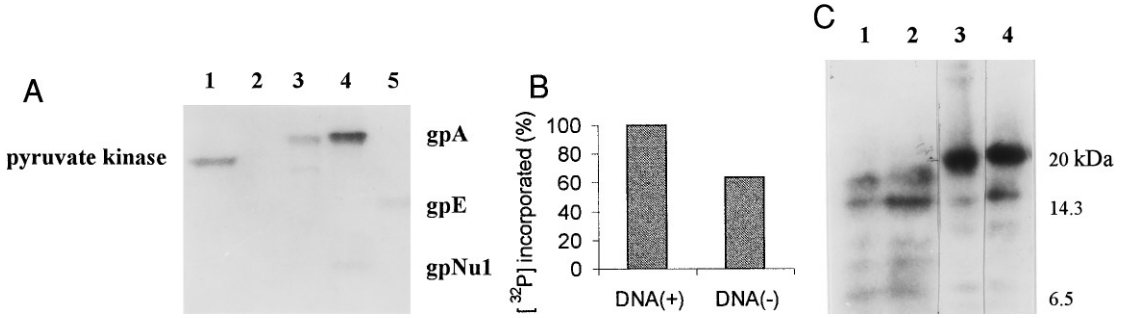


FIG. 4. Photoaffinity labeling with 8-azidoATP. (A) 4 μg of gpA, gpNu1, and pyruvate kinase and 5 μg of terminase and proheads were photolabeled with [α^{-32}P]8-N_3ATP, electrophoresed on a 12.5% SDS–polyacrylamide gel, and autoradiographed. Lane 1, pyruvate kinase; lane 2, gpNul; lane 3, gpA; lane 4, terminase; lane 5, proheads. (B) Terminase was photolabeled in the absence or the presence of 0.1 mg/ml λ DNA, electrophoresed on a 12.5% SDS–polyacrylamide gel, and analyzed using the Phosphorimager. (C) Partial trypsin digests of [α^{-32}P]8-N_3ATP-labeled terminase and gpA were electrophoresed on a 16.5% T and 6% C tricine gel. Lane 1, gpA; lane 2, gpA + λ DNA; lane 3, terminase; lane 4, terminase + λ DNA.

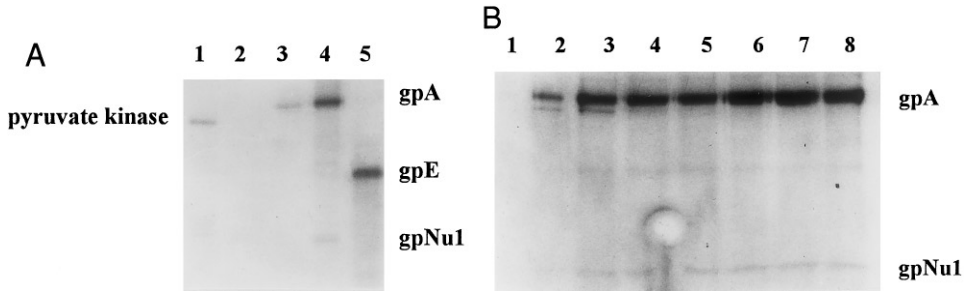


FIG. 5. Photoaffinity labeling with [β,γ^{-32}P]2-N_3ATP. (A) 4 μg of gpA, gpNu1, and pyruvate kinase and 6 μg each of terminase and proheads were photolabeled with 150 μM [β,γ^{-32}P]2-N_3ATP, electrophoresed on a 12.5% SDS–polyacrylamide gel, and autoradiographed. Lane 1, pyruvate kinase; lane 2, gpNul; lane 3, gpA; lane 4, terminase; lane 5, proheads. (B) Time course of photolabeling of terminase by [β,γ^{-32}P]2-N_3ATP. Lane 1, 0 min; lane 2, 15 s; lane 3, 30 s; lane 4, 1 min; lane 5, 2 min; lane 6, 5 min; lane 7, 8 min; lane 8, 10 min of photoactivation.

[β,γ^{-32}P]2-N_3ATP was found to be initially faster than that of ATP for terminase and almost similar for gpA. About 20% of ATP and 80% of [β,γ^{-32}P]2-N_3ATP was hydrolyzed by terminase (10 nM) in the first 10 min. About 50% of ATP and 60% of [β,γ^{-32}P]2-N_3ATP was hydrolyzed by gpA (10 nM) in the first 10 min. Similar, but slightly lower rates of hydrolysis were also observed for 8-N_3ATP (data not shown).

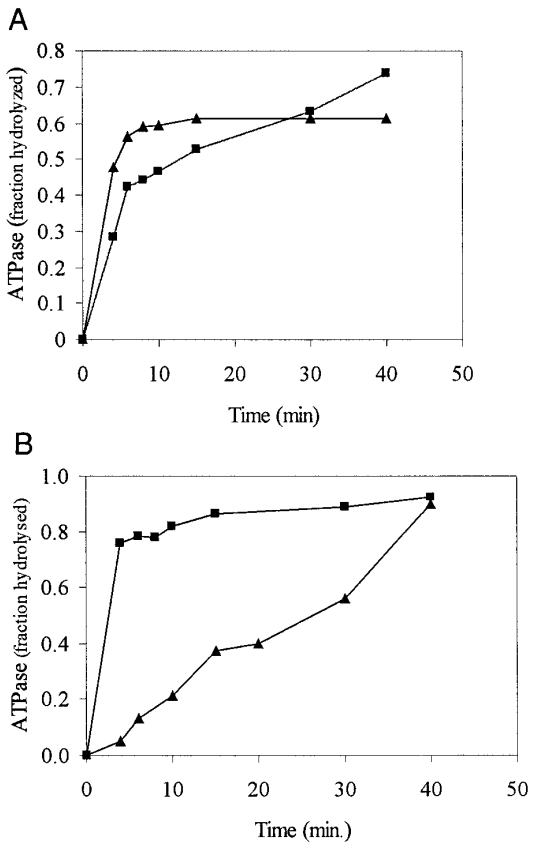


FIG. 6. Hydrolysis of ATP and 2-N_3ATP by gpA (A) and terminase (B). Reaction mixtures contained 20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 7 mM β-mercaptoethanol, 5 mM spermidine, gpA (0.08 mg/ml), or terminase (0.16 mg/ml) and (■) 2-N_3ATP, (0.1 mM), or (▲) [^{14}C]ATP (0.1 mM). Aliquots (2 μl) were withdrawn at the indicated times, applied to polyethyleneimine TLC plates, and developed as described under Materials and Methods. Data points are mean values obtained from two independent experiments.

To confirm that photoinsertion was occurring within the active site, it was demonstrated that photolabeling was both saturable and was protected by ATP. Figure 7A shows the results from an experiment in which terminase was labeled by increasing concentrations of [β,γ^{-32}P]2-N_3ATP and then electrophoresed on a 12.5% SDS gel. The dried gel was quantified using the Phosphorimager. GpA and gpNu1 both exhibit saturation at about 200 μM 2-N_3ATP. In Fig. 7B, terminase, proheads, gpA, and gpNu1 were incubated with 0.5 mM ATP and then photolabeled with 8-N_3ATP. ATP was shown to protect against photoinsertion, demonstrating that the labeling seen was specific for ATP binding sites.

The stochiometry of photoincorporation of 2-azidoATP was determined as shown in Fig. 7C. It was determined that 0.66 mol of 2-azidoATP was incorporated per mole of terminase. As a control the same experiment was performed but with the addition of 1 mM ATP in the reaction. ATP was found to inhibit the photoincorporation of [β,γ^{-32}P]2-N_3ATP, demonstrating that photolabeling was specific for ATP binding sites.

To determine whether the presence of λ DNA had any effect on the binding and crosslinking of 8-azidoATP, terminase was photolabeled in the absence and the presence of 0.1 mg/ml λ DNA (details in legend, Fig. 4). Analysis of Phosphorimager scans of dried gels showed that labeling of gpA was about 15% higher in the presence of DNA. This made it important to determine if additional sites in terminase were being labeled when photolabeling was performed in the presence of DNA. To answer this question, partial trypsin digests of terminase and gpA labeled with [α^{-32}P]8-N_3ATP in the presence or the absence of λ DNA were performed and the peptides resolved on a 16.5% T, 6% C tricine SDS gel and autoradiographed. As shown in Fig. 4C, the tryptic patterns look similar. This indicates that the increased crosslinking seen in

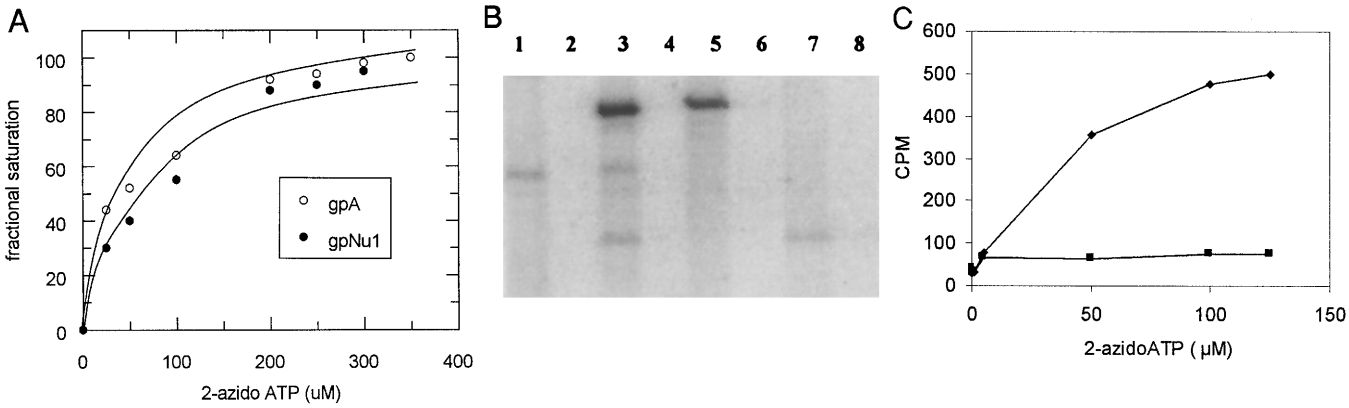


FIG. 7. (A) Saturation of [β,γ^{-32}P]2-N_3ATP photoinsertion into terminase. Terminase was UV photolabeled with increasing concentrations of [β,γ^{-32}P]2-N_3ATP and then electrophoresed on an SDS–polyacrylamide gel. The dried gel was quantified using the Phosphorimager. (B) Protection against photolabeling by [α^{-32}P]8-N_3ATP. Terminase, proheads, gpA, and gpNu1 were incubated with and without 0.5 mM ATP for 10 min, then photolabeled with 8-N_3ATP, and electrophoresed on an SDS–polyacrylamide gel. The dried gel was autoradiographed. Lanes 1, 3, 5, and 7 are proheads, terminase, gpA, and gpNu1. Lanes 2, 4, 6, 8 are proheads, terminase, gpA, and gpNu1 photolabeled in the presence of ATP. (C) Saturation of [β,γ^{-32}P]2-N_3ATP photoinsertion into terminase. Terminase (2.5 μg) was photolysed for 5 min in 20 μl of reaction buffer with the indicated concentrations of [β,γ^{-32}P]2-N_3ATP with (■) or without (◆) ATP. ^{32}P incorporation was determined by filter binding assays as described in Materials and Methods.

the presence of DNA was probably not due to labeling of additional sites in terminase.

Effect of photolabeling by 8-azidoATP on *in vitro* packaging activity

In the absence of the photoprobe, irradiation with UV for up to 2 min did not have any harmful effect on the packaging activity of terminase or gpNu1. On the other hand, UV irradiation for 2 min reduced the packaging activity of gpA to about 10%. Terminase and gpA, when photolysed for 2 min in the presence of increasing concentrations of 8-azidoATP, showed a dose-dependent inactivation (Fig. 8B). However, in an experiment where terminase or gpA was incubated with the highest concentration of 8-azidoATP and UV irradiation was omitted (data not shown), no difference in the packaging activity was observed. Thus the inactivation observed must have resulted from the interaction of the UV-activated photoprobe with the ATP binding site. When concatameric λ DNA was included in the packaging reaction instead of processed DNA, identical results were obtained. Maximal inhibition of the packaging activity of terminase and gpA was observed at 500 μM 8-azidoATP. Under similar experimental conditions the packaging activity of gpNu1 was not affected.

Effect of photolabeling by 8-azidoATP on *in vitro* ATPase activity

The *in vitro* ATPase activity of terminase was reduced to 28% and that of gpA to 20% of their original activity at the highest concentration of 8-azidoATP that was tested (shown in Fig. 8A).

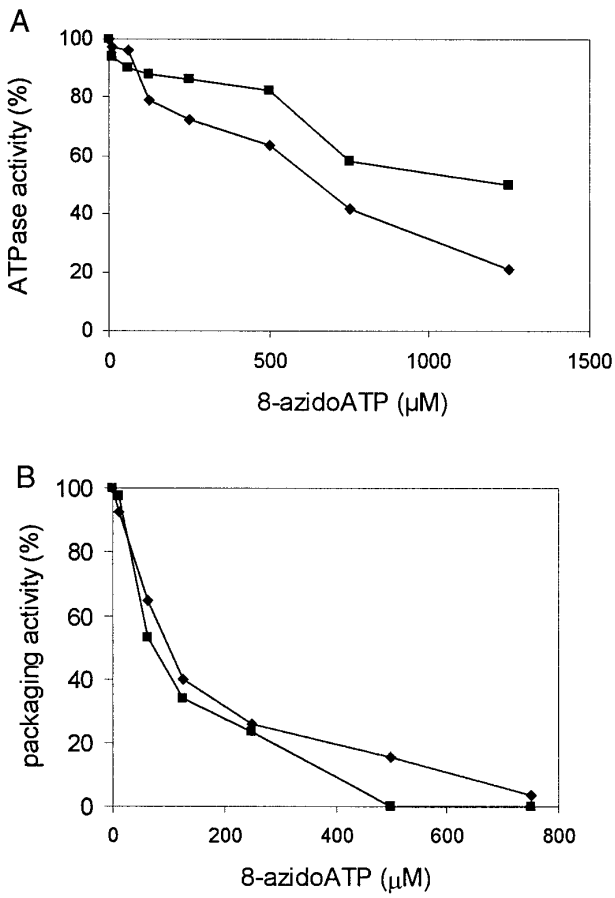


FIG. 8. (A) Inhibition of ATPase and packaging activity of terminase and gpA by photoaffinity labeling with 8-azidoATP. Terminase, 4 μg (◆), and gpA, 5 μg (■), were photolabeled with UV for 2 min, in a volume of 20 μl, with the indicated concentrations of 8-azidoATP. Each reaction was then divided into two and assayed for (A) ATPase activity and (B) packaging activity. Data points are mean values obtained from two independent experiments.

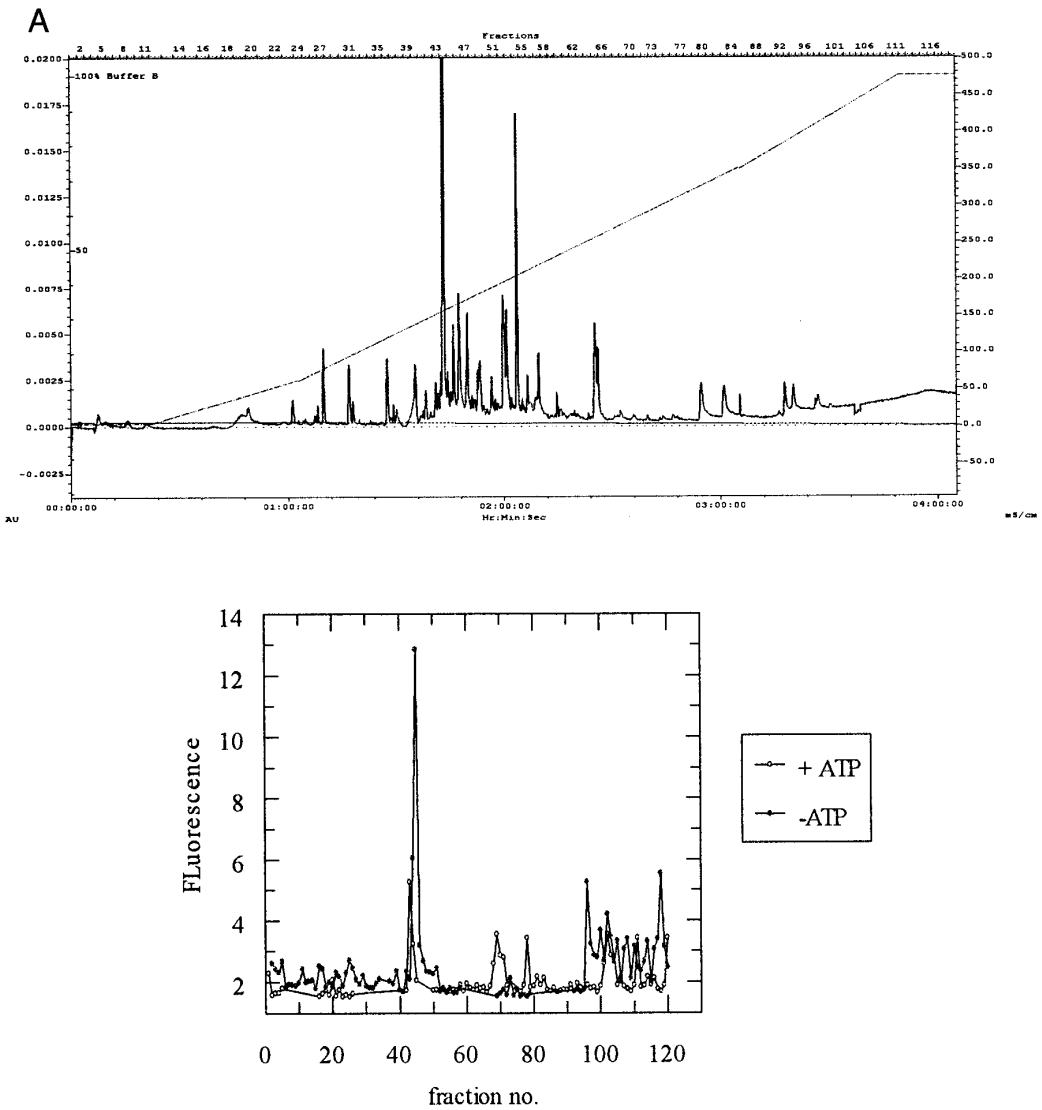
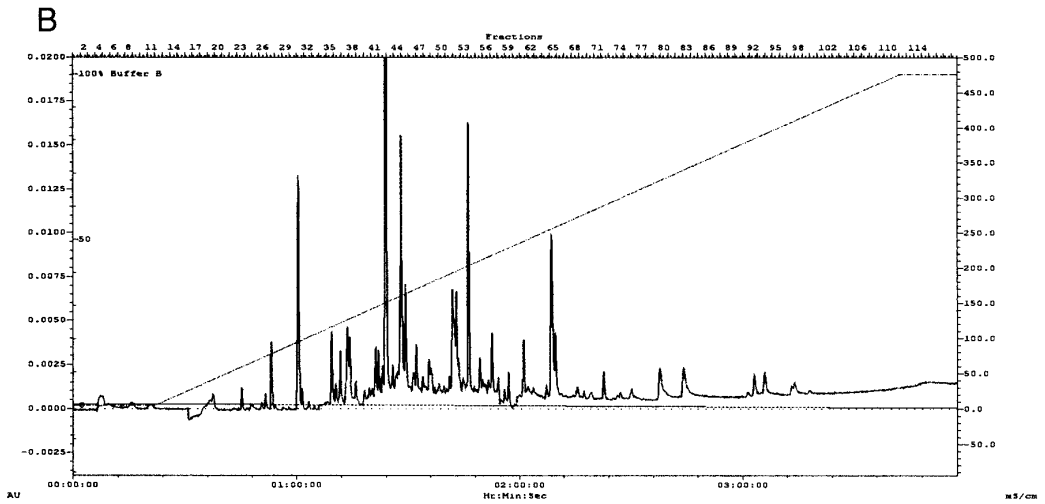


FIG. 9. Purification of FITC-labeled peptides from terminase (A), gpA (B), and gpNu1 (C). Trypsin digests of FITC-labeled proteins were chromatographed on a C_4 column and eluted with a gradient of 80% acetonitrile + 0.1% TFA. One-milliliter fractions were collected and each fraction was measured for fluorescence (excitation at 495 and emission at 524 nm). The fluorescent peptide from trypsin digest of terminase eluting at 32% solvent B was further purified by HPLC by running a gradient of 10 to 40% solvent B in 70 min.

Purification of FITC-labeled peptides

In order to determine the FITC binding sites in FITC-modified terminase, gpA and gpNu1 were extensively digested by trypsin. Labeling was performed in the presence and the absence of ATP. The trypsin digests were fractionated by reverse-phase HPLC and each fraction was monitored for fluorescence. Tryptic digests of terminase yielded one single FITC-labeled peptide which eluted at 32% solvent B (Fig. 9A). Labeling of this peptide was shown to be competitively inhibited when the modification was done in the presence of ATP. This peptide was further purified by HPLC (see legend to Fig. 9). The N terminal sequence of this peptide was determined to be A I M N A M. Determination of the mass localized this peptide to a region from amino acids 59 to 79 in the N-terminal of gpA.

A tryptic digest of gpA, when resolved by reverse-phase chromatography, yielded two fluorescent peaks. Amino acid composition of the second peak, which eluted toward the end of the acetonitrile gradient, revealed that it was not a peptide. The sequence of the first peak was found to be A I M N A M G S D (59—). The mass of this peptide showed it to be the same peptide as the one isolated from terminase. Labeling of this peptide was also shown to be competitively inhibited when FITC modification was done in the presence of ATP (Fig. 9B). A tryptic digest of modified gpNu1 when separated by reverse-phase HPLC yielded one major peak at 35% solvent B (Fig. 9C). The N-terminal sequence of this peptide was determined to be T I Q N W Q E Q G M P V L R (18–) and its mass indicated it to be from amino acids 18 to 35 in the N-terminal of gpNu1 Labeling of this



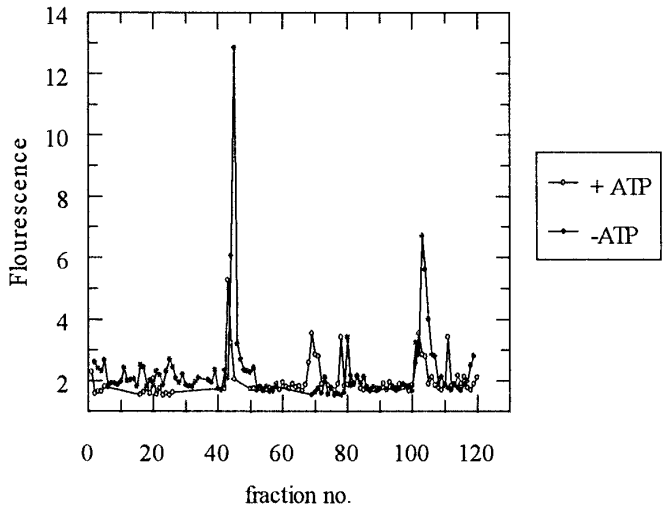


FIG. 9—*Continued*

peptide was shown to be competitively inhibited when FITC modification was done in the presence of ATP.

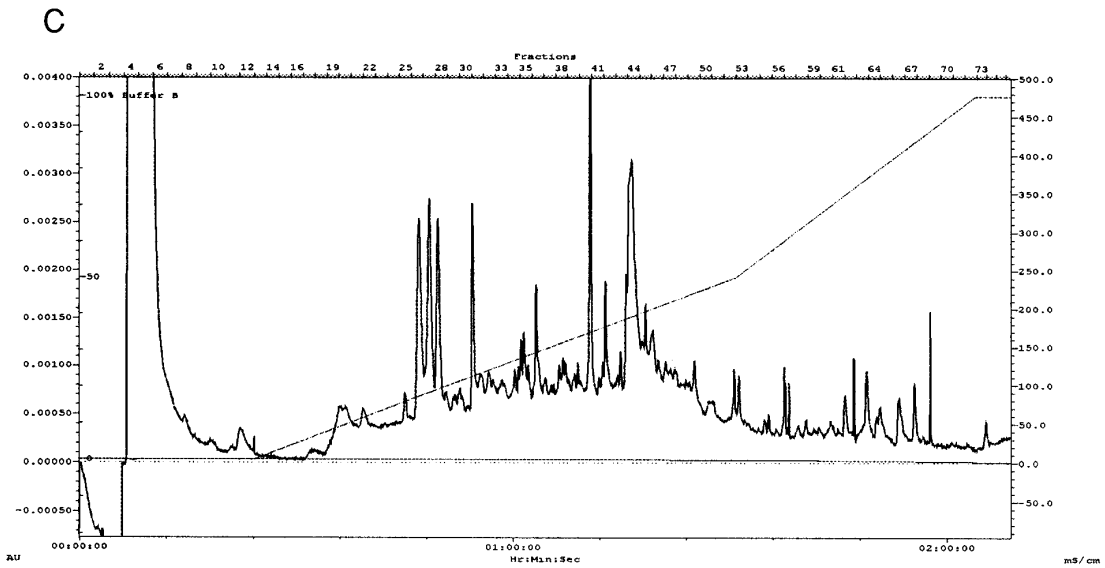
Purification of 8-azidoATP-labeled peptides

Purification of labeled peptides from a tryptic digest of terminase and gpA by reverse chromatography, using acetonitrile as the solvent, did not yield any radioactive peptides.

In order to map the 8-azidoATP binding site, partial cyanogen bromide digests of gpA and gpNu1 were electrophoresed on a 16.5%, T 6% C tricine gel and blotted onto PVDF membranes. Peptide mapping (Fig. 10) indicated that the modified region extended from amino acids 61 to 85 in the N-terminal region of gpA. Peptide E, with an approximate molecular mass of 7 kDa, had greater incorporation of radioactivity than the other peptides. This could possibly be because of labeling of a greater number of residues in peptide E, thus making the peptide run more slowly. Thus, it is possible that peptides E and F are the same but with a different number of residues being labeled. The peptides G and F were cut from the PVDF membrane and N-terminally sequenced. The N-terminal sequence of peptide G was found to be MEVNKKQLADIFGASIRTIQ (1–). Further sequencing was not possible as the number of PTH derivatives released was too low to be detected. In the same way, the N-terminal sequence of labeled peptide F from gpA was found to be GSDYIR(65–).

Purification of 2-azido-labeled peptides by reverse-phase chromatography

Reverse-phase separation of tryptic digests of labeled gpA and gpNu1 did not yield any stable peptides when the separation was done at pH 2 or at 6.5. However, when a tryptic digest of [β,γ^{-32}P]2-N_3ATP-labeled terminase was run on a C_4 column, a single radioactive peak



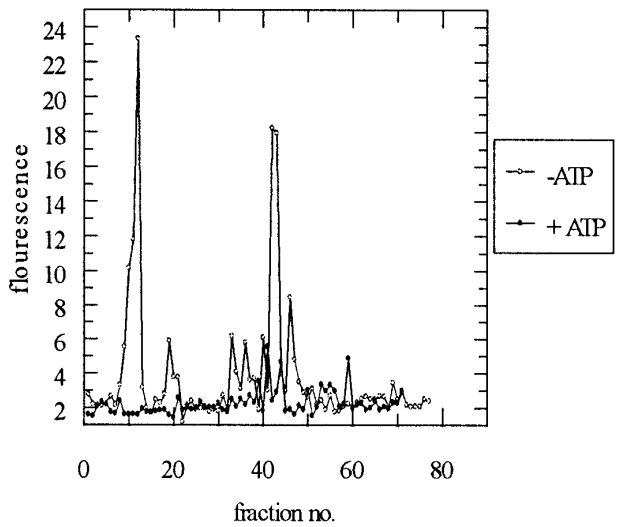


FIG. 9—*Continued*

was observed which eluted at 36% solvent B. The majority of the counts did, however, elute in the flow-through. The N-terminal sequence of this labeled peptide was determined to be VG∗SK, corresponding to the sequence VGYSK (81–84). No PTH derivative was detected during the third cycle. The mass of this peptide was roughly the same as that of the peptide VGYSK modified at one residue by 2-azidoATP as above.

DISCUSSION

ATP plays multiple roles in the different reactions catalyzed by terminase. It acts as an allosteric effector in the nicking reaction. Since poorly hydrolyzable analogs of ATP can substitute for ATP to terminase to ensure the accuracy of the endonucleolytic cleavage, this implies that ATP binding and not hydrolysis is required at this step (Higgins *et al.,* 1988). Strand separation of the annealed ends formed after *cos* cleavage has been shown to require ATP hydrolysis (Higgins *et al.,* 1988; Rubinchik *et al.,* 1994b). Translocation of DNA into the proheads (for review see Black, 1995) is also believed to be powered by ATP hydrolysis. About 2 bp of DNA have been shown to be packaged per molecule of ATP hydrolyzed (ϕ29—Guo *et al.,* 1987; T3—Morita *et al.,* 1993). In view of the multiple interactions that occur between ATP and terminase, it is quite possible that different ATP-reactive sites in terminase might be activated at different stages in the packaging reaction. The *in vitro* ATPase activity of purified terminases is much lower than the ATPase activity thought to be required for packaging *in vivo.* However, Rubinchik *et al.* (1995) showed that *in vitro,* the rate of ATP hydrolysis by terminase was sufficient to account for

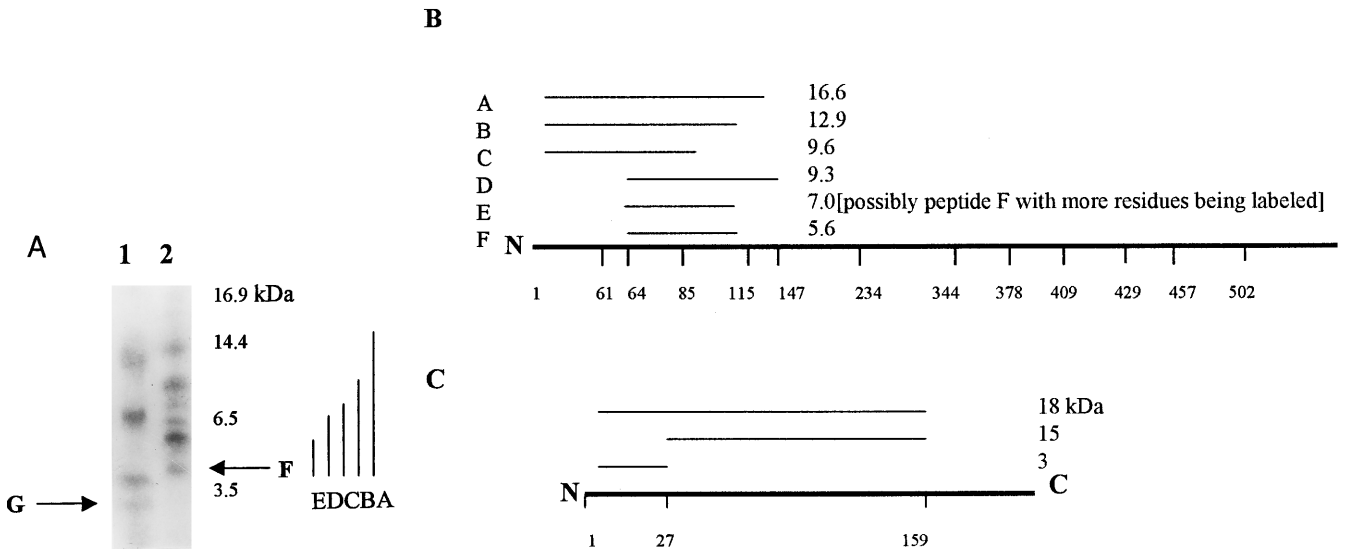


FIG. 10. (A) Partial CNBr digests of [α^{-32}P]8-N_3ATP-labeled gpA (lane 1) and gpNu1 (lane 2) were electrophoresed on a 16.5% T, 6% C tricine gel, transferred to a PVDF membrane, and autoradiographed. (B and C) Peptide mapping of overlapping labeled peptides from gpA and gpNu1. The positions at which methionine residues are present in gpA and gpNu1 have been marked.

the rate of translocation of DNA into proheads in a purified system. This raises the question of which components of the packaging machinery contribute to this ATPase activity and how the hydrolysis of ATP is coupled to the movement of DNA into the head. To begin to answer this question we have used the technique of affinity labeling. Reactive nucleotide analogs allow specific labeling of essential amino acids residues that participate in substrate binding or catalysis. The regions containing the nucleotide-binding domain can be identified by isolating the modified peptide. Furthermore, to find if there are any shared ATP-reactive sites between gpA and gpNu1, labeling of the subunits and the holoenzyme was performed under native conditions and sequences of the labeled peptides from terminase, gpA, and gpNu1 were compared.

FITC is an affinity analog of ATP which has been successfully used in the covalent and irreversible modification of purine nucleotide binding sites of many ATPases and GTPases (Phillips, 1988; Pardo and Slayman, 1988; Pavela-Vrancic *et al.,* 1994). It is a lysine/arginine-specific analog. Positively charged residues are known to play an important role in ion-pair interaction with the negatively charged phosphoryl groups. Since FITC is similar to the hydrophobic purine ring, modification with it allows identification of Lys/Arg residues that interact with the α- phosphoryl group. The 2- and 8-azidoATP are some of the other commonly used affinity analogs of ATP. These photosensitive substrates have been successfully used in labeling the active sites of many purine nucleotide binding proteins (Knight and McEntee, 1985; Cross *et al.,* 1987; Julin and Lehman, 1987; Davis *et al.,* 1990; Salvucci *et al.,* 1992; Grammer *et al.,* 1993; Pavela-Vrancic *et al.,* 1994; Olcott *et al.,* 1994; Bramhall *et al.,* 1997). Irradiation with ultraviolet light converts them into highly reactive nitrenes. These nitrenes can covalently insert into the nearby amino acid chain of the polypeptide backbone. Thus, once the protein is labeled, the region(s) containing the nucleotide binding site can be identified by isolating the modified peptide(s). Some proteins can accept only 2-azidoATP as substrate. This is because 2-azidoATP has the anti-conformation about the N-glycosidic bond (characteristic of ATP), while 8-azidoATP has the syn conformation induced by substitution at the C-8 position. Thus, use of 2-azidoATP offers the advantage of labeling and studying those nucleotide binding sites not accessible to other photoaffinity probes or which have a low affinity for ATP. However, the use of these analogs is limited by the lability of the labeled photoprobe during separation of peptides by reverse-phase HPLC. Affinity labeling with FITC and 2- and 8-azidoATP was used to modify terminase and its subunits gpA and gpNu1 and proheads at their ATP binding sites. These three analogs facilitate identification of those peptides that are in close proximity to the adenine ring of ATP.

X-ray crystallographic studies (Fletterick *et al.,* 1975) have shown that the fluorescein moieties of fluorescein derivatives when bound to the nucleotide binding site of enzymes occupy the adenosine binding regions within the sites. This might also be true in the case of terminase, where labeling with FITC is shown to inactivate the enzyme's ATPase and packaging activity, probably by preventing binding of ATP. The difference in the inhibition of packaging activity of terminase and gpA might be because of a difference in the conformation of the ATP-reactive center of gpA when present as an isolated subunit and when present as a holoenzyme in associa-

tion with gpNu1. GpA is modified four times better than gpNu1 on a weight basis; this is indicative of a higher affinity reactive site. The results show that the site of modification in gpA when part of the holoenzyme terminase is the same as when gpA is labeled alone. The sequence of the labeled peptide based on its mass and N-terminal sequence is AIMNAMGSDYIREVNVVKSAR (amino acids 59–69) with either R-70 or K-76 being the sites of modification. It is possible that the pattern of labeling in gpA and in terminase might be different. If K-76 or R-70, whichever is the essential residue for ATPase activity, is labeled in terminase, then the labeling in gpA is at the other residue. If labeling in gpA occurs predominantly at the residue, which is nonessential for ATPase activity, this would explain why labeling of gpA with FITC does not completely inhibit its packaging or ATPase activity. Interactions of gpA and gpNu1 when they are present together in the holoenzyme probably introduce some alteration in the conformation of the ATP binding domain of gpA. The N-terminus of gpA has also been shown genetically to interact with the C-terminus of gpNu1 (Frackman *et al.,* 1984, 1985). That interaction with gpNu1 stabilizes gpA is supported by the fact that the N-terminus of purified gpA is more labile and susceptible to cleavage upon storage (Parris *et al.,* 1994).

Similarly, the site of labeling in gpNu1 is TIQNWQEQGMPVLRGGGK (amino acids 18–35), with probably R31 as the site of covalent modification with FITC. Residues 27–35 form the consensus sequence of the putative ATP binding domain of gpNu1. K35 has earlier been proposed (Yang, 1993; Hwang *et al.,* 1996) to be the "invariant lysine" of this "P-loop" or phosphate binding motif. Mutations at this position in other ATPases such as RecBCD (Korangy and Julin, 1992) and MutS protein (Haber and Walker, 1991) result in complete loss or reduction of their ATPase activity. It is possible that the ATP binding domain in gpNu1 is formed by a contiguous stretch of amino acids.

The low level of inhibition in packaging activity of FITC-labeled gpNu1 compared to labeled terminase could be explained on the basis of a low-affinity ATP binding site in gpNu1. Binding of ATP to this site may be important for binding of gpNu1 to *cosB,* while the ATPase activity of this site may not contribute significantly to the ATPase turnover required for translocation. Mutations in this segment of the enzyme, especially L40F, are known to act as suppressors of *cos* mutations by strengthening gpNu1 *cosB* interactions (Cai *et al.,* 1997).

No inhibition of the *in vitro cos* cleavage activity of terminase and gpA was observed even when the proteins were modified with FITC concentrations as high as 1 mM (data not shown). Some nonspecific endonucleolytic activity observed in these assays was found to be due to the presence of DMF. Modification with FITC was found to inhibit this nonspecific cutting of the DNA substrate (the *cos* cleavage assay was performed under high-stringency conditions). This indicates an interaction between the ATP binding and endonucleolytic domains of terminase similar to the one observed by Rubinchik *et al.,* (1994b). When concatameric DNA was used for the *in vitro* packaging assays, results identical to those when processed DNA was used were obtained.

Crystal structures of other ATPases such as F_1-ATPase have confirmed the presence of residues photolabeled by 2-azidoATP at the nucleotide binding site (Abrahams *et al.,* 1994). That the binding and labeling occur specifically at the ATP binding site was demonstrated by (1) the ability of 2- and 8-azidoATP to serve as substrates and (2) the complete protection afforded by ATP against photolabeling by 2- and 8-azidoATP. Results from photoaffinity labeling experiments using 2- and 8-azidoATP clearly demonstrate the presence of a single high-affinity ATP-reactive center in gpA and a low-affinity ATP-reactive center in gpNu1. GpE, a capsid protein, was also shown to be labeled by these two analogs. This suggests a possible role of the proheads in the ATPase activity associated with the translocation of λ DNA. It is interesting to note that in the ϕ29, T3, and T7 packaging systems, ATPase activity was observed only in the presence of proheads, DNA, and both the subunits of terminase. This is in contrast to λ where each of the subunits displays an *in vitro* ATPase activity independent of proheads, which have no ATPase activity of their own.

Photoaffinity labeling with 8-azidoATP was shown to inhibit both the packaging and the ATPase activity of terminase and gpA. This is indicative of the modification of active site residues. The greater extent of labeling seen in subunits when photolabeled as a holoenzyme compared to when labeled as separate subunits could be explained on the basis of protein–protein interactions which could possibly affect the affinity of the ATP-reactive sites of the two subunits. Comparisons of the ATP binding sequences in terminase and other ATPases, which have been identified by use of 2- and 8- azido ATP, show considerable similarity, although a consensus sequence was difficult to deduce. 2-N_3ATP was found to be the better analog for labeling the proteins as well as for isolation of the labeled peptide. Similar results using these two photoprobes have been observed for F_1-ATPase (van Dongen *et al.,* 1986). This might be attributed to the "anti"-conformation in which 2-azidoATP exits. A single 2-azido-modified peptide, VGY∗SK (amino acids 81–85), was isolated from terminase. Its sequence localized it to the same region in gpA as did FITC- and 8-azido-labeled peptides. In most of the studies where these azido analogs have been used to identify ATP binding sites, photolabeled peptides that have been isolated usually involve photoinsertion into a tyrosine residue (Cross *et al.,* 1988; Kim and Haley, 1991). The same regions in gpA and gpNu1 were consistently identified by using three different affinity analogs. The region identified in gpA when labeled as a subunit is the same as the

one when it was labeled as a part of the holoenzyme. This indicates that gpA does not undergo major structural changes when it enters into association with gpNu1 to form terminase in terms of its ATP binding site; however, some alterations may take place which change its packaging domain.

Experiments using affinity analogs such as pyridoxal 5′phosphate, pyridoxal 5′-diphospho-5′ -adenosine, and 5′-*p*-fluorosulphonylbenzoyl-adenosine have already been initiated to identify those residues in terminase that interact with the β and γ phosphoryl groups of ATP.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids overproducing the holoenzyme pCM101, the subunits gpA and pCM2, and gpNu1, pCM35 have been described by Chow *et al.* (1987). The extracts from the strain *Escherichia coli* OR1264, transformed by the plasmid pCM230 which is a source of proheads, gpD, gpW, gpFI, tails etc. (Chow *et al.,* 1987), were used to assay the *in vitro* packaging activity of gpNu1. Crude extracts of NS428 (Murialdo *et al.,* 1987) which provide proheads, gpD, gpW, gpF1, and gpNu1 were used for assaying the *in vitro* packaging activity of terminase and gpA.

Chemicals and enzymes

FITC (isomer I) and CNBr were from Sigma. [2$-^{14}$C]ATP was from Amersham. Trypsin (1-1-tosylamido-2-phenylethyl chloromethyl ketone treated) was from Worthington. ATP was purchased from Boerhinger Mannheim. [α^{-32}P]8-N_3ATP was from ICN. 2-N_3AMP (generously provided by Dr. Richard L. Cross) was enzymatically phosphorylated to [β,γ^{-32}P]2-N_3ATP as described by Melese and Boyer (1985) and purified by anion exchange chromatography as described by Bramhall *et al.* (1997). The purity of the synthesized product was confirmed by TLC (Czarnecki, 1984). The [β,γ^{-32}P]2-N_3ATP migrated as a single spot on thin-layer cellulose plates with an R_f value of 0.23 using 1-butanol:glacial acetic acid:water (5:2:3, v/v) as solvent system. The fractions containing [β,γ^{-32}P]2-N_3ATP were lyophilized directly and then redissolved into water and stored at -70°C. It had a specific activity of 10^{14} cpm/mol. As 2-azidoATP tautomerizes into photoinsensitive tetrazolo and azido forms (Czarnecki, 1984) at the pH under which the reactions were conducted, about 50% of the photo analog was determined to be in the azido form. The concentration and specific activity of [β,γ^{-32}P]2-N_3ATP were determined once equilibrium of the azido and the tetrazolo isomers had been reached at pH 7, using extinction coefficients of 10.3 and 7.0 mM^{-1} at 271 and 310 nm, respectively (Czarnecki, 1984). Acetonitrile (HPLC grade) was from Caledon Labs.

Purified proteins

The proteins were purified as described by Parris *et al.* (1994). Proteins were more than 95% pure judged by Coomassie blue staining of proteins separated by SDS–polyacrylamide gel electrophoresis. The protein concentrations were determined by Bradford's method using the Bio–Rad protein assay kit from Bio–Rad, following the manufacturer's instructions.

Covalent modification of terminase, gpA, and gpNu1 by FITC

The reaction buffer contained 20 mM Tris (pH 8.0), 5 mM $MgCl_2$, and 50 mM NaCl. The indicated concentration of FITC was added from a fresh stock in dimethylformamide (DMF) yielding a final DMF concentration of 2.5%. The control reactions where FITC was omitted also contained 2.5% DMF. Incubation with terminase or gpA or gpNu1 was performed in the dark at room temperature for half an hour in experiments assaying the effect of FITC modification on the different enzymatic activities of the proteins. The labeled proteins were then separated from the unbound FITC by gel filtration on 1-ml Sephadex G-50 columns equilibrated in 20 mM Tris (pH 8.0) and 7 mM β-mercaptoethanol.

Preparative labeling and proteolysis of the proteins

Terminase (150 μg), gpA (100 μg), or gpNu1 (100 μg) was incubated in the dark with 0.5 mM FITC in 20 mM Tris, pH 8.0, 50 mM NaCl, and 5 mM $MgCl_2$ in the presence or the absence of 5 mM ATP at 37°C for 45 min. The reaction volume was 250 μl. Proteins were precipitated by addition of trichloroacetic acid to a final concentration of 10%. After 20 min on ice the precipitate was collected by centrifugation and the pellet washed twice with 1 ml of 80% (v/v) acetone (-20°C). After briefly drying under vacuum, the pellet was dissolved in 45 μl of 0.4 M ammonium bicarbonate, containing 8 M urea. This was then diluted with 180 μl of water to yield a 2 M final urea concentration. An aliquot was added from a freshly dissolved stock of trypsin to give a final trypsin to protein ratio of 1:20. The trypsinization was carried out at 37°C for 18–20 h.

Photoaffinity labeling of terminase and its subunits with [α^{-32}P]8-N_3ATP and [β,γ^{-32}P]2-N_3ATP

Four to five micrograms of terminase, gpA, and gpNu1 was incubated with the appropriate concentrations of photoprobe on ice, in the dark, for 10 min in a buffer containing 20 mM Tris, pH 8.0, 5 mM $MgCl_2$, and 50 mM NaCl with or without 0.1 mg/ml λ phage DNA. The samples were then irradiated with a handheld 254-nm Mineralite UVS-11 UV lamp for 2 min from a distance of 4 cm at 4°C. The reactions were quenched by addition of DTT to 10 mM and then electrophoresed on a 12.5% SDS–

polyacrylamide gel as described by Laemmli (1970). The gels were either autoradiographed or exposed to phosphor storage screens and scanned using the Phosphor Imager (Molecular Dynamics). In experiments where unlabeled 8-azidoATP was used to determine the effect of photolabeling on the enzymatic activities, the reactions were desalted on 1-ml Sephadex G-50 columns equilibrated in 20 mM Tris (pH 7.5) and 7 mM β-mercaptoethanol and the photolabeled proteins were assayed for packaging or ATPase activity.

Stochiometry of labeling with 2-azidoATP

Terminase was incubated with different concentrations of [β,γ^{-32}P]2-N_3ATP in the absence and the presence of 5 mM ATP and photolysed in a total volume of 20 μl as described above. The proteins were precipitated in the presence of 10 μg of bovine serum albumin by addition of 200 μl of 10% TCA. These were spotted on Whatman GF/A filters using a vacuum assembly. The filter was washed twice with 5% TCA and once with 100% ethanol and dried under a heat lamp. Covalently incorporated radioactivity was determined in a scintillation counter using Cytoscint as the scintillant.

Packaging assays

The crude extracts were prepared and the packaging assays were performed as described by Parris *et al.* (1994). In a gpNu1 complementation assay 5 μl of an extract of OR1265 transformed by pCM2 was added as a source of gpA. The packaging reaction was started by the addition of FITC-modified proteins and then incubated for 1 h at room temperature. The phage particles produced from the packaging reactions were titered on QD5003*sup* F^+ (Yanofsky and Ito, 1966) and the results expressed as PFU/ml reaction mixture.

ATPase assays

ATPase assays were performed using [2-^{14}C]ATP as described by Rubinchik *et al.* (1994b). The reaction volume was 10 or 20 μl. The assay was performed in a buffer containing 20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 5 mM spermidine, 7 mM β-mercaptoethanol, 0.1 mg/ml double-stranded DNA, and 10 mM ATP and initiated by the addition of FITC-modified proteins. The products of the reaction were analyzed as described previously (Gold and Becker, 1983; Parris *et al.,* 1994).

Isolation of FITC-labeled peptides by reverse-phase chromatography

This was performed on the Biologic chromatography system from Bio–Rad. The tryptic digests were lyophilized, dissolved in solvent A (0.1% TFA), and applied to a C_4 column (RP-304, 250 $\times$ 4.6 mm, from Bio–Rad). The peptides were eluted with a gradient of solvent B (80% acetonitrile, 0.1% TFA) at a flow rate of 0.5 ml/min as follows: for terminase and gpA, 100% solvent A for 20 min was followed by a linear gradient of 0 to 100% solvent B for 200 min and then an isocratic flow of 100% solvent B for 20 min. For gpNu1, the peptides were eluted with an isocratic flow of 100% solvent A for 20 min followed by a linear gradient of 0 to 100% solvent B for 120 min and then an isocratic flow of 100% B for 10 min. Detection was carried out by monitoring the absorbance at 280 nm. Each fraction was measured for fluorescence on a Fluorolog 2 series spectrofluorometer (excitation at 495 and fluorescence emission at 524 nm). The peak fractions were concentrated and repurified on the C_4 column using a shallower gradient.

Isolation of [α^{-32}P]8-N_3ATP-labeled peptides from PVDF membrane

Photolabeled gpA and gpNu1 were precipitated in 10% TCA and washed twice with acetone (−20°C). The protein pellets were air dried and resuspended in 250 μl of a CNBr solution (100 mg/ml, w/v in 70% formic acid). The reaction was allowed to proceed for 90 min at room temperature and then diluted four times with distilled water, freeze dried twice, and then resuspended in sample buffer and electrophoresed on 16.5% T, 6% C tricine SDS separating gel (Schaager and Van Jagow, 1987). The peptides were then electrophoretically transferred on to Immobilon-P^{sq}, PVDF membrane (Millopore). The tank transfer was carried out at 80 V for 1 h. The membrane was stained according to manufacturer's instructions and autoradiographed.

Purification of 2-azido-labeled peptide from terminase

Terminase (200 μg) was incubated with 300 μM of [β,γ^{-32}P]2-N_3ATP and photolysed as described before. Proteolysis and reverse-phase chromatographic separation was as described for purification of FITC-labeled peptides. Each fraction was counted for radioactivity. The peak fractions were concentrated on Speed Vac and submitted for sequencing.

Peptide sequencing and mass spectrometric analysis

Peptide sequencing was performed by the Protein Analysis Service, Department of Biochemistry, University of Toronto, using an Applied Biosystems 470A gas-phase Sequenator coupled to a Model 120A phenylthiohydantoin- derivative analyzer. Mass spectrum analysis was performed by the Carbohydrate Research Centre, University of Toronto.

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