The location of the interaction of the COOH terminus of the bacteriophage T4 DNA polymerase with its trim- eric, circular sliding clamp has been established. A pep- tide corresponding to the COOH terminus of the DNA polymerase was labeled with a fluorophore and fluo- rescence spectroscopy used to show that it forms a specific complex with the sliding clamp by virtue of its low KG value (7.1 ± 1.0 μM). The same peptide was labeled with a photoaffinity probe and cross-linked to the sliding clamp. Mass spectrometry of tryptic digests determined the sole linkage point to be Ala-159 on the sliding clamp, an amino acid that lies on the subunit interface. These results demonstrate that the COOH terminus of the DNA polymerase is inserted into the subunit interface of its sliding clamp, thereby conferring processivity to the DNA polymerase.

Multiprotein complexes lie at the heart of DNA replication. In bacteriophage T4, the DNA polymerase (gp43), its processivity factor or sliding clamp (gp45), and the clamp-loader (gp44/62) form the holoenzyme that is loaded onto DNA in a multistep process requiring hydrolysis of ATP (1–3). gp44/62 (a 4:1 complex of gp44 and gp62) is only a transient species in the holoenzyme assembly process, hydrolyzing ATP to load gp45 onto DNA and then chaperoning gp43 onto the gp45/DNA complex (4). Likewise, the intermediate states of assembly are distinct and transient (3, 5). The x-ray crystal structures of gp45 from bacteriophage T4 and gp43 from bacteriophage RB69 (65% identity with gp43 from T4 (6)) are known (7), but the precise relative orientations of the proteins during and after the holoenzyme assembly process are unknown. X-ray crystallography should be able to eventually determine the structures of stable multiprotein complexes such as the final holoenzyme structure, but other methods will be required to determine the structures of transient or unstable structures during the holoenzyme assembly process. We have chosen to use fluorescence spectroscopy and mass spectrometry to map the connection point between the COOH terminus of gp43 and gp45, the interaction that is at the core of the holoenzyme and confers processivity to gp43 (8).

We have shown previously that the COOH terminus of gp43 is essential for forming the holoenzyme, and a peptide corre- sponding to the COOH terminus of gp43 inhibits the interac- tion of gp45 and gp44/62, thereby inhibiting formation of the holoenzyme (8). The importance of the COOH terminus of gp43 was also investigated by others (9). Additionally, the COOH termini of gp33 and gp55 have been shown to be essential for interaction withgp45 (10). gp33 is a transcriptional co-activator (11), and gp55 is a σ-family protein (12), and their interaction with gp45 is required in late RNA transcription (13). The sequences of the COOH termini of gp93, gp55, and gp43, as well as the sequence of the COOH-terminal peptide of gp43 (peptide 1), are shown in Fig. 1. These three proteins have a common (S/T)/LDFL motif, suggesting a shared mode of interaction with gp45. gp45 has shown to be a circular trimer by x-ray crystallography, with an internal hole large enough to accommodate DNA. We have recently found that the trimer is open in solution and have speculated that the COOH-terminal tail of gp43 would be inserted into the open subunit interface of gp45 during holoenzyme assembly (14).

In this study, Peptide 1 was labeled with the fluorophore IAEADS or the photoaffinity probe TFPAM-3 (see Fig. 1) to investigate the interaction point on gp45. The peptide 1-IAE- DANS conjugate was shown to interact with gp45 in a 1:1 complex (peptide/gp45 monomer) with micromolar affinity, suggesting a specific interaction. The peptide 1-TFPAM-3 con- jugate was cross-linked to gp45, which was then digested with trypsin and the resultant peptides separated by HPLC and analyzed by mass spectrometry. We have found that the COOH-terminal peptide of gp43 cross-links to Ala-159 of gp45, an amino acid that sits at the subunit interface of gp45, confirming that the COOH terminus of gp43 is inserted into the gp45 subunit interface to form the topological link between gp43 and the DNA template.

EXPERIMENTAL PROCEDURES

Materials and Their Sources—Peptide 1 was a generous gift of Mi- chael Moore of SmithKline Beecham. IAEADS and TFPAM-3 were from Molecular Probes (Eugene, OR). Trifluorooracetic acid (HPLC grade) was from J.T. Baker (Phillipsburg, NJ). Trypsin (t-1-tosylamide-2-
phenylethyl chloromethyl ketone-treated) was from U.S. Biochemical Corp. The MALDI matrix α-cyan-4-hydroxycinnamic acid was from Sigma. All other materials were of analytical grade or better.

Peptide Labeling—To form the peptide 1-IADENS conjugate, 1 mg of peptide 1 was dissolved in 500 μl of 50 mM HEPES, pH 7.6, 150 mM potassium acetate, 1 mM EDTA, and 10% glycerol (labeling buffer) and mixed with 5 μg of IADENS dissolved in 500 μl of labeling buffer. The mixture was nitrated in the dark at 4 °C for 12 h and then purified by HPLC on a 4.6 × 250-mm C18 column (Vydac, Hesperia, CA) using the following gradient at 1 ml min⁻¹: solvent A, 0.1% trifluoroacetic acid in H₂O; solvent B, 0.1% trifluoroacetic acid in CH₃CN; the column was equilibrated with 80% solvent A, and 350 μl of the above mixture was injected and the column maintained at the initial conditions until stabilization of the baseline. The peptide was eluted with a linear 13-min gradient from 80 to 54% solvent A. The fraction containing the peptide conjugate was dried under vacuum, redissolved in water, and quantitated using 5660 m⁻¹ cm⁻¹ as the extinction coefficient of the probe at 336 nm. To form the peptide 1-TFPAM-3 conjugate, 0.5 mg of peptide 1 were dissolved in 500 μl of labeling buffer and mixed with 1 μl of 50 mM dithiothreitol to reduce any intermolecular disulfides. TFPAM-3 (1 mg) was dissolved in 200 μl of dimethyl sulfoxide and added to the above mixture. After incubation in the dark on ice for 1 h, the conjugate was purified by HPLC on a 4.6 × 250-mm C18 column (Vydac) using the following gradient at 1 ml min⁻¹: solvent A, 0.1% trifluoroacetic acid in H₂O; solvent B, 0.1% trifluoroacetic acid in CH₃CN; 100% solvent A for 2 min, a 20-min linear gradient from 100 to 50% solvent A, and a 5-min linear gradient from 50 to 0% solvent A. The peptide 1-TFPAM-3 conjugate was collected at 25 min, dried under vacuum, redissolved in 50 mM Tris, pH 7.5, and quantitated using 14,000 m⁻¹ cm⁻¹ as the extinction coefficient of the probe at 250 nm. Modification was confirmed by MALDI-MS ([M + H]⁺ m/z 1272.8, calculated 1272.5).

Protein—gp45 was purified as described previously (18) and quantitated using a Bradford protein assay (Bio-Rad).

Fluorescence—Fluorescence measurements were performed on a SLM-8000C spectrofluorimeter (SLM Instruments, Urbana, IL). gp45 and peptide 1-IADENS were in 25 mM Tris acetate, pH 7.5, 150 mM potassium acetate, 10 mM magnesium acetate, and 10 mM β-mercaptoethanol were excited at 280 nm and FRET observed at 485 nm (titrating in gp45) or tryptophan quenching observed at 340 nm (titrating in peptide 1-IADENS). Binding data were fit to the equation Fobs = F₀ − (1/Tₚ ) × ΔF, where F₀ is the observed fluorescence, Fₚ is initial fluorescence, T is the concentration of the compound (gp45 or peptide 1-IADENS) that was titrated into the solution, and ΔF is the change in observed fluorescence from initial fluorescence. Data were fit using KaleidaGraph (Synergy Software, Reading, PA) to determine Kᵢ,ₜ.

Photocross-linking and Tryptic Digests—gp45 was dialyzed versus 20 mM HEPES, pH 7.0, 50 mM NaCl, and 1 mM EDTA to remove DTT and tryptophan mode. Post-source decay spectra were obtained using the addition of an 80% solvent A, and a 5-min linear gradient from 50 to 0% solvent A. The peptide 1-TFPAM-3 conjugate was collected at 25 min, dried under vacuum, redissolved in 50 mM Tris, pH 7.5, and quantitated using 5660 m⁻¹ cm⁻¹ as the extinction coefficient of the probe at 250 nm. Modification was confirmed by MALDI-MS ([M + H]⁺ m/z 1272.8, calculated 1272.5).

RESULTS

We recently determined that the COOH terminus of gp43 is critical for forming the bacteriophage T4 holoenzyme (8). The COOH terminus of gp43 shares homology with the COOH termini of gp33 and gp55 (see Fig. 1), proteins that also have been shown to interact with gp45 through their COOH termini (10). We have determined the contact point of gp45 on the COOH terminus of gp43 using fluorescence spectroscopy and mass spectrometry.

Fluorescence—An eight-amino acid peptide (Peptide 1, see Fig. 1) corresponding to the seven COOH-terminal amino acids of gp43 and an NH₂-terminal cysteine was labeled with the fluorophore IADENS (Fig. 1). Titration with gp45 (constant peptide concentration) resulted in a FRET signal to IADENS at 485 nm upon exciting gp45 tryptophans at 280 nm (Fig. 2). Likewise, titration with peptide (constant gp45 concentration) resulted in gp45 tryptophan quenching at 340 nm upon exciting at 280 nm (Fig. 2). These fluorescence titrations were fit to an average Kᵢ,ₜ value of 7.1 ± 1.0 μM (8.2 ± 0.4 μM and 6.1 ± 0.5 μM for titrating peptide and gp45, respectively). To fit both curves successfully with the same Kᵢ,ₜ value, the gp45 concentration had to be in terms of monomers rather than trimers, suggesting that there are three peptide binding sites per gp45 trimer. The absence of sigmoidal character indicates the absence of cooperative binding and the low Kᵢ,ₜ value suggests a specific interaction.

Photocross-linking—Peptide 1 was modified with a photoaffinity probe to determine the interaction point of the COOH terminus of gp43 on gp45. Several photoaffinity probes were tested for their ability to form photocross-links between peptide 1 and gp45. Derivitizing the NH₂ terminus of peptide 1 with an azide resulted in quenching of the photoactivated azide by the neighboring free thiol of cysteine. Derivitizing the cysteine thiol with a standard azide resulted in a low yield of photocross-links, while the perfluoroarylazide TFPAM-3 (Fig. 1) gave the highest yield of cross-links. Following UV irradiation of gp45 and peptide 1-TFPAM-3, cross-linked gp45 showed a slight decrease in mobility by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Using 15 equivalents of peptide per gp45 trimer, about 50% of gp45 was shifted to this new band (data not shown). Unreacted, photolyzed peptide was almost completely removed by ultrafiltration and the mixture...
of cross-linked and uncross-linked gp45 was digested with trypsin. HPLC separation of the tryptic digest yielded about 20 peaks, including several late-eluting peaks that did not appear in a mock-photocross-linking reaction with gp45 but without peptide 1-TFPAM-3. All peaks were collected and subject to analysis by mass spectrometry.

**Mass Spectrometry**—The identities of the tryptic peptides were initially confirmed by MALDI-MS. The majority of possible tryptic fragments of gp45 were identified (13 of 21) as well as tryptic fragments due to one missed tryptic cleavage. The eight tryptic fragments that were not observed were predicted to be less than 500 or greater than 5000 Da. The late eluting peaks not observed in the mock-photocross-linking reaction were assigned to the photolysis products of peptide 1-TFPAM-3, which did not react with gp45, or unphotolyzed peptide 1-TFPAM-3. These species were not completely removed by ultrafiltration before treatment with trypsin. One HPLC peak (20.5 min) was found to contain two tryptic fragments of gp45 as well as a fragment that did not have a mass corresponding to a tryptic fragment of gp45 or a fragment missing one or more predicted tryptic cleavages. This fragment, *m/z* 2362 (Fig. 3), corresponds to the mass of the gp45 tryptic fragment from amino acids 155–164 plus the mass of peptide 1-TFPAM-3 expected following photolysis. The specificity of product formation leads us to conclude that this tryptic fragment of gp45 lies at the subunit interface. The fragment has one missed cleavage at Arg-162 and is 1 Da higher in mass than expected. Exact-mass MALDI-MS using internal calibration standards confirmed the mass assignment (data not shown). We speculate that Arg-162 may have been oxidatively converted to citruline during the photocross-linking process. This would add one mass unit to the total mass of the product and prevent cleavage by trypsin at this amino acid.

The 20.5-min HPLC peak was subjected to post-source decay in the MALDI using an ion gate set to allow input of ions *m/z* 2362 ± 50 in order to confirm the identity and structure of the cross-linked product and determine the exact linkage point of the photocross-linker. Fig. 4 shows the assigned structure of the cross-linked product as well as several diagnostic fragment ions from the post-source decay experiment. The only amino acid in the 155–164 tryptic fragment that remains attached to the photocross-linking moiety in all ion fragments is Ala-159. These ion fragments therefore allow us to assign the sole linkage point of the photocross-linker as Ala-159, as shown in Fig. 4. All of the ion fragments are also consistent with the predicted amino acid sequence of the 155–164 gp45 tryptic fragment.

**DISCUSSION**

DNA replication and late RNA transcription in bacteriophage T4 both require the coordination of several proteins to form processive complexes. The role of gp45 is central in these processes, providing a topological link between proteins and nucleic acid. Defining the exact mode of interaction between the individual proteins in these complexes will further the understanding of the structure and function of these multiprotein complexes.

The COOH termini of gp43 (8), as well as gp33 and gp55 (10), have been shown previously to be essential for interaction with gp45. The motif (S/T)LDFL is found at the COOH termini of all three of these proteins (see Fig. 1) and suggests a common mode of interaction with gp45. We previously employed a mutant of gp43 lacking the last six COOH-terminal amino acids and found that this mutant could not be assembled into a holoenzyme complex. Peptide 1 was shown to be a competitive inhibitor of the ATPase activity of gp44/62 in the presence of gp45 with a *Kᵢ* of 1.6 μM (expressed as a function of gp45 trimers; multiply by 3 to compare with *Kᵢ* below), thereby inhibiting the formation of the holoenzyme (8). The peptide
1-IAEDANS conjugate was found to have a $K_i$ of 2.9 $\mu M$ (expressed as a function of gp45 trimers; data not shown), indicating that modification of peptide 1 at the terminal cysteine does not significantly alter its ability to disrupt the interaction between gp44/62 and gp45.

We have confirmed the specific interaction of peptide 1 with gp45 by fluorescence titration. Peptide 1 was labeled with the fluorophore IAEDANS, and both FRET from gp45 tryptophans to IAEDANS and gp45 tryptophan quenching by IAEDANS were observed (see Fig. 2). The stoichiometry of the peptide-gp45 interaction was found to be one peptide to one gp45 monomer, since gp45 concentrations had to be expressed as a function of monomers to obtain the same $K_D$ value (7.1 $\pm$ 1.0 $\mu M$) for both titrations. The low $K_D$ value, as well as the low $K_i$ value, suggest that the peptide is making a specific interaction with gp45. The presence of a specific interaction improves the chances that photocross-linking will occur at predominantly one site and thereby improves the odds that the photocross-linked product will be found and characterized.

It is unlikely in vivo or in vitro that three gp43 molecules bind to one gp45 molecule, however. In the model of the holoenzyme shown in Fig. 5, there is not enough room for even two gp43 molecules to bind on the same face of gp45 (gp43 interacts with gp45 on only one face of gp45 (19)), and the stoichiometry of gp43 and gp45 in the holoenzyme has been shown to be 1:1 (20). The small size of the peptide probably causes each gp45 subunit interface to behave as if isolated from the other two. The absence of sigmoidal character in the binding curves is supportive of the gp45 subunit interfaces binding the peptide without cooperativity.

The peptide 1-TFPAM-3 conjugate was found to cross-link to gp45 at Ala-159.3 an amino acid that lies at the gp45 subunit interface. Photocross-links were formed with an excess of peptide 1-TFPAM-3 to improve the yield of cross-links. A mixture of cross-linked and uncross-linked gp45 was digested with trypsin, the tryptic fragments separated by HPLC, and the identities of the tryptic fragments assigned by MALDI-MS analysis. Only one cross-linked fragment was found in the 20 HPLC peaks assayed: amino acids 155–164 connected to peptide 1-TFPAM-3 (see Fig. 4). This tryptic fragment has a missed cleavage at Arg-162 and the ion found, $m/z$ 2362, is 1 mass unit higher than predicted. Oxidative damage of Arg-162 would prevent trypsin from cleaving at this amino acid, so we speculate that Arg-162 has been converted to citrulline. Conversion of the charged guanidine moiety to the uncharged urea moiety adds 1 Da in mass. Citrulline is the product of arginine oxidation by nitric-oxide synthetase (21), and the photocross-linking

![FIG. 4. Structure of cross-linked product.](image)

![FIG. 5. Model of the holoenzyme using the bacteriophage RB69 gp43 (left, darker) and bacteriophage T4 gp45 (right, lighter) x-ray crystal structures and B-form DNA. The COOH-terminal tail of gp43, indicated with an arrow, has been inserted into the gp45 subunit interface.](image)
conditions (strong UV light and highly reactive nitrenes) would be conducive to generation of reactive oxygen species and oxidation of proteins.

The location of cross-linking, Ala-159, was determined from the identification of fragmentation ions using post-source decay in the MALDI instrument. Fig. 4 shows the assigned structure of the cross-linked product as well as several diagnostic fragment ions that allow us to confirm Ala-159 as the sole linkage point. The \( m/z \) 1719 fragment ion results from loss of LTRVK, the five amino acids neighboring on the COOH-terminal side of Ala-159, while the \( m/z \) 1014 fragment ion is also diagnostic for the Ala-159 linkage point. Unfortunately, the resolution of the MALDI during post-source decay (empirically determined to be less than \( \pm 2 \) Da) does not allow us to unambiguously assign the Arg-162 oxidation to citruline. We are currently performing additional mass spectrometry experiments to characterize this oxidation. These experiments as well as the full details of the mass spectrometry experiments will be reported in a subsequent report.

Fig. 5 shows a model of the holoenzyme based on the x-ray crystal structures of bacteriophage RB69 gp43 (7) and bacteriophage T4 gp45.1 Threading the tail of gp43 through the subunit interface of gp45 and docking the two proteins against each other allows gp43 to contact predominantly two subunits of gp45. Determining the precise orientation of these other contacts will require additional experiments. gp33 and gp55, whose structures are unknown, likely interact with gp45 in a fashion similar to gp43, with their COOH termini inserted into the gp45 subunit interface, due to the homology of their COOH termini (see Fig. 1).

Conclusion—The COOH terminus of gp43 has been shown by fluorescence spectroscopy and mass spectrometry to be inserted into the gp45 subunit interface. DNA replication and late RNA transcription achieve their processivity by the interaction of the COOH termini of gp43, gp33, and gp55 with the subunit interface of gp45, providing substantial stabilization of the complexes by relatively short stretches of amino acids. Photocross-linking and mass spectrometry should prove to be powerful tools, providing insight into the structures of transient multiprotein complexes.

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