Structural Analyses of gp45 Sliding Clamp Interactions during Assembly of the Bacteriophage T4 DNA Polymerase Holoenzyme

I. CONFORMATIONAL CHANGES WITHIN THE gp44/62-gp45-ATP COMPLEX DURING CLAMP LOADING*

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A multisubunit ring-shaped protein complex is used to tether the polymerase to the DNA at the primer-template junction in most DNA replication systems. This “sliding clamp” interacts with the polymerase, completely encircles the DNA duplex, and is assembled onto the DNA by a specific clamp loading complex in an ATP-driven process. Site-specific mutagenesis has been used to introduce single cysteine residues as reactive sites for adduct formation within each of the three subunits of the bacteriophage T4-coded sliding clamp complex (gp44). Two such mutants, gp44S19C and gp44K81C, are reacted with the cysteine-specific photoactivable cross-linker TFPAM-3 and used to track the changes in the relative positioning of the gp44 subunits with one another and with the other components of the clamp loading complex (gp44/62) in the various stages of the loading process. Cross-linking interactions performed in the presence of nucleotide cofactors show that ATP binding and hydrolysis, interaction with primer-template DNA, and release of ADP all result in significant conformational changes within the clamp loading cycle. A structural model is presented to account for the observed rearrangements of intersubunit contacts within the complex during the loading process.

The complexities of genomic DNA replication place special demands on the multiprotein complex that must carry out this process. The complex must move processively along the DNA template to account for the rapid and efficient synthesis seen in vivo (1, 2) but must also be able to cycle on and off the DNA rapidly to form Okazaki fragments during lagging strand synthesis. It is probably the complexity of this semidiscontinuous synthesis of double-stranded DNA, as well as the need to interact with repair, recombination, and transcription complexes, that has resulted in the evolution of separate protein subassemblies to perform different replication functions. This separation of functions may make it easier for the system to respond to rapidly changing requirements during the replication process (3).

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Processive DNA synthesis is not an intrinsic quality of the T4 DNA polymerase itself under physiological solution conditions. On the contrary, under these conditions T4 DNA polymerase dissociates from single-stranded template DNA after only a few nucleotide residues have been added to the nascent DNA strand (4). Instead, another protein complex, called a sliding clamp, is used to fix the polymerase to the DNA and permit the addition of many nucleotide residues per binding event to the nascent chains within the replication fork. These sliding clamps, in the various replication systems, have the characteristic shape of a ring that can completely encircle the double-stranded DNA (5, 6).1 Thus, the term “sliding clamp” (8) signifies that polymerases bound to these proteins are “clamped” to the DNA but are still free to “slide” along the DNA templates in the course of elongation synthesis.

The loading of a sliding clamp onto DNA requires the ATP-driven action of yet another protein complex. ATP hydrolysis is probably necessary to produce the substantial conformational changes that must occur when a topologically closed ring is forced to encircle DNA. In the T4 system, the sliding clamp is a homotrimeric ring of gp45 subunits (9, 10), and the ATPase “clamp loader” is the multisubunit gp44/622 complex, which contains four gp44 and one gp62 subunit (9). The arrangement of the gp44 and gp62 subunits within the clamp-loading complex is not yet known. The gp44 subunits carry the ATP binding sites of the complex as well as a binding site for the DNA cofactor that can stimulate the ATPase activity of both the gp44/62 complex and of a tetrameric complex of gp44 alone (10, 11). The gp62 subunit is required for further stimulation of the ATPase activity by the gp45 sliding clamp, which does not stimulate the ATPase activity of the complex formed by gp44 subunits alone (11). The four gp44 subunits bind four ATP molecules, which appear to be hydrolyzed in a single turnover reaction during the clamp loading process (12, 13). The rate of ATP hydrolysis correlates well with the rate of holoenzyme complex formation (about 1 s−1; see Ref. 14), implying that ATP hydrolysis is, at least partially, rate-limiting within the clamp loading reaction (12, 13). The gp44/62 complex functions catalytically to load the gp45. Therefore, once the gp45 loading process is complete, gp44/62 dissociates from the gp45-DNA complex and is free to load another clamp (12, 13, 15, 16). ATP is absolutely required to assemble a functional holoen-

1 I. Moarefi and J. Kuriyan, manuscript in preparation.

2 The abbreviations used are: gp44/62, the complex of the protein subunits to one gene 62 protein subunit; gp44, the trimer of the protein product of T4 gene 45; gp41, the hexamer of the protein product of T4 gene 41; gp44S19C, gp44K81C and gp45T7C, gp45 mutants in which the serine in position 19, the lysine in position 81, and the threonine in position 7, respectively, have been mutated to cysteine; TFPAM-3, N-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidopropionamide; ATP·S, adenosine 5′-O-(3-thiophosphate); MOPS, 3-(N-morpholino)propanesulfonic acid.

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zyme onto DNA, and a nonhydrolyzable ATP analog (ATPγS) cannot substitute for it (17, 18). Footprinting (19) and cross-linking (20) studies have shown that ATPγS is nonetheless sufficient to assemble the accessory proteins complex onto primer-template DNA. These studies have shown that ATP hydrolysis causes a rearrangement of the proteins, together with a decrease in the DNA contacts of all three proteins of the complex. However, little is known about the actual physical changes that occur within the loading process during ATP hydrolysis to lead to the assembly of the gp45 trimer ring onto the DNA duplex.

Direct studies of the conformational changes within the gp45 protein that are induced by the ATPase activity of the gp44/62 have recently been performed in our laboratory (21) and in the Benkovic laboratory (22) by fluorescently labeling single cysteine residues introduced in gp45 by site-directed mutagenesis at position 19 (21) and at position 7 (22). These studies both showed that ATP hydrolysis is required to induce a fluorescent intensity change in the labeled gp45 mutants in the presence of gp44/62 and that this fluorescent change is reversed upon the addition of primer-template DNA, apparently reflecting the dissociation of the gp44/62-gp45 complex as a consequence of clamp loading. The binding of ATPγS does not induce this fluorescence change. Titration of the fluorescently labeled gp45 mutants with gp44/62 in the presence of ATP revealed a binding stoichiometry of one gp45 trimer to one gp44/62 complex. This result suggested that the observed fluorescence change reflects the formation of an activated clamp loading complex between the two protein subassemblies. Depolarization of fluorescence studies supported this conclusion, revealing a larger anisotropy value and therefore a larger size of the rotational unit when ATP was present, compared with the anisotropy observed in the absence of this nucleotide cofactor or in the presence of ATPγS or ATP and DNA together (21). We note that ATPγS has been shown to induce a DNA-dependent conformational change (22), which is consistent with previous studies in which a DNA-accessory protein complex was formed in the presence of ATPγS (19, 20), although it is not known if this complex is properly assembled.

To gain more insight into the modes and dynamics of contact between gp45 and the gp44/62 complex, we have labeled the same gp45 mutants used in the previous fluorescence work with a bifunctional reagent containing a maleimide group at position 19 (21) and at position 7 (22). These studies both showed that ATP hydrolysis is required to induce a fluorescent intensity change in the labeled gp45 mutants in the presence of gp44/62 and that this fluorescent change is reversed upon the addition of primer-template DNA, apparently reflecting the dissociation of the gp44/62-gp45 complex as a consequence of clamp loading. The binding of ATPγS does not induce this fluorescence change. Titration of the fluorescently labeled gp45 mutants with gp44/62 in the presence of ATP revealed a binding stoichiometry of one gp45 trimer to one gp44/62 complex. This result suggested that the observed fluorescence change reflects the formation of an activated clamp loading complex between the two protein subassemblies. Depolarization of fluorescence studies supported this conclusion, revealing a larger anisotropy value and therefore a larger size of the rotational unit when ATP was present, compared with the anisotropy observed in the absence of this nucleotide cofactor or in the presence of ATPγS or ATP and DNA together (21). We note that ATPγS has been shown to induce a DNA-dependent conformational change (22), which is consistent with previous studies in which a DNA-accessory protein complex was formed in the presence of ATPγS (19, 20), although it is not known if this complex is properly assembled.

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**EXPERIMENTAL PROCEDURES**

**Materials**—The cysteine-specific photo-cross-linker TFPAM-3 (described in Ref. 23, and shown as Structure 1) was a generous gift of Dr. R. Aggeler from the laboratory of Dr. R. A. Capaldi at the University of Oregon. This compound is now available from Molecular Probes, Inc. (Eugene, OR).

Perfluorophenyl azides have been shown to be better photolabeling agents than their nitrophenyl azide analogues, because substitution of ring protons with fluorine atoms in phenyl azides leads to fewer rearrangement reactions and higher yields of insertions into C–H and N–H bonds (24).

A polyclonal antiserum to the gp44/62 complex was generously supplied by Dr. E. P. Geiduschek of the University of California at San Diego, and a polyclonal antiserum to gp62 was the generous gift of Dr. M. Reddy (University of Wisconsin, Milwaukee). DNA oligonucleotides were purchased from Genosys (The Woodlands, TX) and were used without further purification. All other biochemicals and chemicals were obtained from Sigma, Aldrich, or Boehringer-Mannheim and were also used without further purification.

**Primer-Template DNA**—The primer-template DNA used in these experiments is shown as Sequence 1 and was made by annealing the component oligonucleotides. The completeness of the hybridization process was confirmed by native gel electrophoresis.

**Proteins**—The construction, by site-directed mutagenesis, of gp45 mutants gp45S19C and gp45K81C, containing a single cysteine for serine substitution at position 19 or a cysteine for lysine substitution at position 81, respectively, has been described (21). Fig. 1 shows the positions within the gp45 ring of the cysteine residues in these two mutants. gp44/62 complexes and wild-type and mutant gp45 were purified as described previously (13). The purification of gp41 helicase has also been described (25). Protein concentrations were determined by UV absorbance at 280 nm, using the following calculated molar extinction coefficients. For gp44/62, ε280 nm = 1.23 × 10⁴ M⁻¹ cm⁻¹; for gp45, ε280 nm = 5.72 × 10⁴ M⁻¹ cm⁻¹; and for gp41, ε280 nm = 4.55 × 10⁴ M⁻¹ cm⁻¹ (27).

**Labeling of gp45 Mutants and Cross-linking Procedures**—The gp45K81C and gp45S19C mutants were passed through BioSpin 6 centrifugation columns (Bio-Rad) equilibrated with 50 mM MOPS buffer (pH 7), 0.5 mM EDTA, and 10% glycerol to remove any thiols and primary amines present in the protein storage buffer. About 50 μl of protein stock solution were labeled at a concentration of 30–40 μM gp45 monomer, using 250 μM TFPAM-3 (diluted from a 4 mM stock solution in Me₂SO) at room temperature for 1 h. After quenching the reaction with 1 mM cysteine for 50 min, excess label was removed by passage through another BioSpin 6 column. The labeled proteins were stored at −20 °C in buffer containing 25 mM HEPES (pH 7.5), 60 mM potassium acetate, 6 mM magnesium acetate, and 25% glycerol.

Labeling of the cysteine residues of both gp45 mutants was complete, as indicated by reaction of the proteins with the thiol-detecting reagent, 5,5'-dithiobis(2,2'-nitrobenzoic acid) (28). Before modification with TFPAM-3, all three cysteine residues of the gp45 trimers in both mutants could be reacted with 5,5'-dithiobis(2,2'-nitrobenzoic acid). No reaction was observed after the mutant proteins were modified with TFPAM-3. Labeled protein concentrations were determined using the BCA protein assay (Pierce) with unlabeled proteins as standards. This was necessary because the protein spectra overlap with that of the TFPAM-3 label.

Replication-stimulating activities of the gp45 mutants are unaffected by the amino acid changes and modifications made (31).

Phosphorylation was carried out in 25 mM HEPES (pH 7.5), 60 mM potassium acetate, 6 mM magnesium acetate, 10% glycerol, at the proteins, DNA, and nucleotide concentrations indicated for each experiment, by irradiating the samples for 45 min at room temperature in the tip of a microcentrifuge tube (1.5 ml) at a distance of about 2 cm from a 6-W "366-nm" UV lamp (UVP, Inc., model UVL-56, Blak-Ray lamp). Under

This "366-nm" UV lamp was used because its putative emission spectrum falls far above the absorption wavelengths for proteins and nucleic acids, and we wished to avoid photochemical damage to these entities. As our data show, this lamp is very effective in inducing cross-linking of our proteins. In addition, the absorption spectrum of the dye chromophore significantly as a function of time of irradiation with this lamp (R. Aggeler, personal communication). On the other hand, as we were reminded by Professor E. Peter Geiduschek (University of California at San Diego), the TFPAM-3 adduct itself absorbs little, if any, light at 366 nm. Thus, the effectiveness of this lamp in inducing photochemical cross-linking probably reflects the breadth of its actual

![Structure 1](image-url)
these conditions the label is completely photolyzed in about 35 min.\(^4\) The cross-linked products were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting, followed by subunit identification using polyclonal antibodies to gp45, gp44/62, and gp62, respectively. Western blotting was carried out in a Bio-Rad TransBlot System using Nitropure-supported nitrocellulose membranes (0.22 \(\mu\)m) purchased from Micron Separations, Inc. (Westboro, MA). Antibodies were visualized by chemiluminescence using an ECL kit from Amersham Corp.

**RESULTS**

Two different mutants of gp45 were constructed by site-specific mutagenesis to introduce single defined cysteine residues as reactive sites within the protein (21). The positions of the cysteines in these mutants are shown in Fig. 1 (drawn according to coordinates kindly supplied by I. Moarefi and J. Kuriyan.\(^5\) In previous work, we had reacted these cysteines with fluorophores to examine the conformational changes induced in the gp45 protein by the action of the gp44/62 ATPase (21).

To better define structural features of the interaction between the two proteins and to monitor their changing relative positions during the loading process, these mutants were reacted with a cysteine-specific photo-cross-linker to permit us to probe near-neighbor contacts at specific positions on the gp45 trimer. The photo-cross-linker (TFPAM-3) used in this work (Ref. 23; see “Experimental Procedures”) contains a maleimide group that can be reacted with a cysteine residue and a tetrafluorophenyl azide group that can be photoactivated to form a highly reactive and relatively nonspecific nitrene that inserts into a nearby chemical bond with high efficiency (24). These functional groups of the cross-linker are separated by a flexible covalent linker that is 13 Å in length. Therefore, when we state in what follows that one protein subunit “contacts” another at a particular gp45 residue position, we mean that the proteins have approached within 13 Å of one another at this position under the defined conditions. The cross-linked products obtained were analyzed using SDS-polyacrylamide gel electrophoresis and Western blotting, as described under “Experimental Procedures.”

**Photo-cross-linking of TFPAM-3-labeled gp45S19C to gp44/62 in the Absence of ATP**—The cysteine residues of the gp45S19C mutant lie on one side of the ring of the gp45 trimer and close to the central hole (Fig. 1). Photolysis of the TFPAM-3-labeled gp45S19C mutant alone (Fig. 2, lane 2) leads to the appearance of a new band that migrates faster than the unphotolyzed sample (Fig. 2, lane 1) and corresponds to an internally cross-linked gp45 subunit. In the presence of gp44/62 (Fig. 2, lane 3), photolysis results in the appearance of three cross-linked products that run above the gp44 subunit and are not visible in the unphotolyzed sample (Fig. 2, lane 4). These products were shown by Western analysis (Fig. 2B, lanes 2, 7, and 12) to correspond to covalent gp45-gp62 adducts (lower band) and two gp45-gp44 adducts (corresponding to the two upper bands, labeled \(a\) and \(b\), that run very close together; the third closely spaced band of this group is a contaminant).

This means that the TFPAM-3 cross-linkers attached to the cysteines located at position 19 on each subunit of the gp45 ring can reach both gp44 and gp62 subunits within the loading complex. The two gp45-gp44 cross-linked products that run with similar mobilities probably result from cross-links formed...
between Cys-19 on the gp45 subunit and different sites on individual gp44 subunits.

**Cross-linking of gp45S19C in the Presence of ATP—**When photolysis is carried out in the presence of ATP, we observe an increase in the yield of the band representing gp45-gp62 cross-linked product, as well as an increase in the upper band (band b) of the two closely spaced gp45-gp44 cross-linked products. The lower band (band a) nearly disappears. Also, a new band of slower mobility (band c) appears that is identified as a new gp45-gp44 cross-linked product by Western analysis (Fig. 2A, lane 4, and Fig. 2B, lanes 3, 8, and 13).

**Cross-linking of gp45S19C in the Presence of ATP and Primer-Template DNA—**When 30/50-mer primer-template DNA (see “Experimental Procedures”) is added to a complex gp44/62 and gp45S19C in the presence of ATP (Fig. 2A, lane 5; Fig. 2B, lanes 4, 9, and 14), the band with the slowest mobility (band c) disappears, and the overall cross-linking pattern resembles that of the two proteins in the absence of ATP and DNA. Western blot analysis (Fig. 2B) confirms this and shows even more clearly the differences in the amount of cross-linking mentioned above, with the amount of the cross-linked products increasing when ATP is added and decreasing again when DNA is added in the presence of ATP.

**Photo-cross-linking of TFPAM-3-labeled gp45K81C in the Absence of ATP—**The gp45K81C mutant carries its cysteine residue very near to the subunit interface along the outside rim
of the gp45 ring (Fig. 1). Not surprisingly, the photolysis of this TFPAM-3-modified mutant (Fig. 3A, lane 2) leads to the appearance of two new bands above the monomer, consistent with the formation of cross-linked gp45 dimer and trimer species (see “Discussion”).

In the presence of gp44/62 (Fig. 3A, lane 3), photo-cross-linking with the gp45K81C mutant generates several extra bands (labeled a, b, c, d, e, and f in Fig. 3), which Western analysis shows to contain gp45 and the gp44 subunits (Fig. 3B, lanes 3, 6, and 10). No cross-linked product involving the gp62 subunit is observed with this mutant under these conditions, implying that TFPAM-3 adducts attached to Cys-81 on the gp45 subunits can only reach the gp44 subunit of the gp44/62 complex.

Cross-linking of gp45K81C in the Presence of ATP—An overall decrease in both the number and the intensity of the gp44-gp45 cross-linked products is observed when cross-linking with gp45K81C is carried out in the presence of ATP. We observe that the fainter bands (bands a and f) disappear (see the Western blot, Fig. 3B, lanes 4 and 7), that band c decreases in intensity, and that the stronger bands (bands d and e) decrease slightly in intensity. More significantly, a new cross-linked product appears that is barely seen by silver staining but is clearly detected by Western analysis. This band results from the cross-linking of gp45 and gp62 subunits (Fig. 3B, lanes 4 and 11). Western analysis allows us to detect three gp45-gp62 cross-linked products. This is expected, even if only one of the three Cys-81 residues in the trimer can reach the gp62 subunit, since two or three gp45 subunits can still be cross-linked to the gp62 subunit via inter-gp45 subunit cross-linking. The above observations suggested that ATP induces a conformational change that weakens the contact of the Cys-81 adduct with the gp44 subunit and allows at least one of the substituted Cys-81 residues to contact the gp62 subunit.

Cross-linking of gp45K81C in the Presence of ATP and Primer-Template DNA—When 30/50-mer DNA containing a primer-template DNA (Fig. 3A, lane 4) is used in the cross-linking reaction, the fainter bands (bands a and f) disappear (see the Western blot, Fig. 3B, lanes 4 and 7), that band c decreases in intensity, and that the stronger bands (bands d and e) decrease slightly in intensity. More significantly, a new cross-linked product appears that is barely seen by silver staining but is clearly detected by Western analysis. This band results from the cross-linking of gp45 and gp62 subunits (Fig. 3B, lanes 4 and 11). Western analysis allows us to detect three gp45-gp62 cross-linked products. This is expected, even if only one of the three Cys-81 residues in the trimer can reach the gp62 subunit, since two or three gp45 subunits can still be cross-linked to the gp62 subunit via inter-gp45 subunit cross-linking. The above observations suggested that ATP induces a conformational change that weakens the contact of the Cys-81 adduct with the gp44 subunit and allows at least one of the substituted Cys-81 residues to contact the gp62 subunit.
template junction is added to the substituted gp45K81C mutant in the presence of ATP, the cross-linking pattern (Fig. 3A, lane 5; Fig. 3B, lanes 8, and 12) resembles in some ways that obtained with ATP alone. Some of the gp45-gp44 cross-linked products seen in the absence of ATP and DNA have disappeared (bands a and f) or are only barely visible (band c). In other ways, this new pattern resembles that obtained with gp44/62 and gp45 alone, in that gp45-gp46 cross-linked products are not present. Also, the two main gp44-gp45 cross-linked products (bands d and e) have comparable intensities. These two bands are slightly more intense in the presence of ATP and DNA or in the absence of both cofactors than in the presence of ATP alone.

Photo-cross-linking of TFPAM-3-labeled gp45K81C to the gp44/62 Complex in the Presence of Various Nucleotide Cofactors—To distinguish between the effects of binding and of hydrolysis of ATP, we performed photo-cross-linking experiments in the presence of different nucleotides. The cross-linking pattern of the TFPAM-3-labeled gp45S19C obtained in the presence of ATP plus EDTA (Fig. 4, lane 5) is very similar to that obtained in the presence of ATP alone (Fig. 4, lane 4), suggesting that the binding of ATP by gp44/62 is sufficient to induce a conformational change. However, the amount of cross-linked products is greater in the presence of ATP with Mg\textsuperscript{2+}, indicating that either the complex is somehow stabilized, leading to more complex formation and a higher cross-linked band intensity, or that a different gp45 conformation is attained after ATP has been hydrolyzed, placing the label closer to the gp44/62 complex and thus resulting in higher cross-linking efficiency. Similar results were obtained by cross-linking in the presence of ATPγS (data not shown).

The direct addition of ADP, a product (with P\textsubscript{i}) of the ATP hydrolysis reaction catalyzed by gp44/62, appears to drive the gp44/62-gp45S19C complex into a still different cross-linked conformation (Fig. 4, lanes 6). This conformation is manifested by the near disappearance of the band with the slowest mobility (band c) that was present in the samples containing ATP and ATP plus EDTA and by the relative amounts of the two closely migrating gp45-gp44 products (the lower band predominates in the presence of ADP, while the upper band predominates when any of the other nucleotide cofactors are present).

Photo-cross-linking of TFPAM-3-labeled gp45K81C to gp44/62 in the Presence of Various Nucleotide Cofactors—Fig. 5 shows the results of cross-linking the labeled K81C mutant of gp45 to gp44/62 in the absence and presence of various nucleotide cofactors. We note the strong similarity of the cross-linking patterns obtained in the presence of ATP (Fig. 5A, lane 4) and in the presence of ATP + EDTA (Fig. 5A, lane 6). This agrees with observations already made with the S19C mutant. In both cases, the gp45-gp44 cross-linked products decrease in intensity to a similar extent relative to the cross-links seen in the absence of added nucleotides (Fig. 5A, lane 3). However, the gp45-gp62 cross-linked products revealed by Western analysis (Fig. 5B) are seen only in the presence of ATP and not in the presence of ATP plus EDTA (or in the presence of any other nucleotide). This suggests that ATP hydrolysis is needed to cause the particular change in the conformation of the loading complex that puts a Cys-81 adduct in contact with the gp62 subunit. We note that the addition of ATPγS and ATP plus EDTA does not lead to a similar cross-linking pattern (compare lanes 5 and 6 in Fig. 5); in particular, the gp44/62 cross-linked products are less intense in the presence of ATPγS. However, the cross-linked trimer band appears to be more intense. This suggests that ATPγS cannot completely mimic the ATP-bound state of the loading complex.

The direct addition of ADP also drives the gp45K81C-containing clamp loader complex into a unique conformation. Although the main gp44-gp45 cross-linked product bands (bands d and e) appear to be very similar in intensity to those in the sample without nucleotides, band c is much less intense in the ADP sample, and bands a and f (which are not visible in the gel shown) are also seen in a more intensively stained gel that allowed detection of these bands in complexes without added nucleotides (data not shown).

Finally, we note that the cross-linking patterns obtained in the presence of ATP and DNA (Fig. 3A, lane 5) and in the presence of ADP (Fig. 5A, lane 7) are quite similar. Both patterns differ from that obtained in the absence of nucleotides and DNA (Fig. 3A, lane 3) in that bands a, b, and f are not present and band c decreases in intensity to a similar extent. Other features also differentiate these patterns from those
obtained in the presence of ATP (Fig. 3A, lane 3 or Fig. 5A, lane 4). Bands d and e are more intense in the presence of ATP and DNA and in the presence of ADP. Furthermore, the gp45-gp62 cross-linked products seen in the presence of ATP alone are not detected in either of these two samples (Fig. 3B, lane 12; Fig. 5B, lane 4).

**DISCUSSION**

We have created mutants of gp45 by site-directed mutagenesis, introducing single cysteine residues as reactive sites for covalent protein modification. In a previous study (21), these mutants were labeled with fluorescent reagents to monitor the interaction of the gp45 sliding clamp with the gp44/62 loading complex during the ATP-dependent loading process. A related study, using a different mutant of gp45, produced very similar results (22). Both studies show that in the presence of ATP (but not in the presence of ATPγS), a complex of gp44/62 with the fluorescently labeled gp45S19C (21) or gp45T7C (22) mutant undergoes a conformational change that is revealed by an increase in fluorescence intensity, together with a blue shift of the emission maximum. Data obtained by titrating fluorescently labeled gp45 mutants with the gp44/62 complex in the presence of ATP revealed a stoichiometry of 1 gp45 trimer to 1 gp44/62 4:1 complex, suggesting that the fluorescence change reflected the formation of a gp45-gp44/62 multiprotein complex (21, 22). Fluorescence anisotropy measurements were in agreement with this hypothesis, since the largest anisotropy value (and therefore the largest rotational size) is observed when the gp44/62 is added to gp45 in the presence of ATP. We also found that the addition of 30/50-mer primer-template DNA was able to reverse, in part, both the observed fluorescence and the observed anisotropy changes (21). This result was consistent with a role for DNA in dissociating gp45 from gp44/62 as a consequence of clamp loading.

In the present study, we have used a photo-cross-linking agent to label these gp45 mutants to investigate near-neighbor contacts of the positions of defined residues in the gp45 trimer ring. The results are in good agreement with the earlier fluorescence studies and provide further insight into the mechanism of the loading process. Both gp45 mutants (gp45S19C and gp45K81C) can be cross-linked to the gp44/62 complex, even in the absence of ATP, suggesting that a gp45-gp44/62 complex is formed under these conditions. We showed that the cross-linking observed is not "collisional," in that TFPAM-3-labeled gp45S19C that was irradiated in the presence of the gp41 helicase hexamer at the same protein concentrations revealed no cross-linked products (data not shown). A small but significant fluorescence anisotropy change was also seen in the absence of nucleotide at ~8-fold lower concentrations of gp45 and gp44/62 complexes.

The Cross-linking Evidence—In the absence of nucleotides labeled Cys-19 can be cross-linked to both the gp44 and gp62 subunits. The presence of a single cross-linked product band containing both gp45 and gp62 subunits (Fig. 2, A, lane 3, and B, lanes 2, 7, and 12) suggests that only one gp45 subunit contacts the single gp62 subunit. The two closely spaced cross-linked product bands containing the gp44 and gp46 subunits (bands a and b in Fig. 2, A, lane 3, and B, lanes 2, 7, and 12) indicate that single gp45 subunits contact gp44 subunits at different positions, giving rise to two bands on the gel.

The overall amount of cross-linked products increases in the presence of ATP (Fig. 2, A, lane 4, and B lanes 3, 8, and 13), which is consistent with the increased number of gp45-gp44/62 complexes formed under these conditions as revealed by fluorescence studies (21). Moreover, a conformational change involving contact of the gp45 with the gp44 subunit is clearly indicated by the disappearance of the lower band of the two closely spaced gp45-gp44 cross-linked products, together with the appearance of a new gp45-gp44 band (Fig. 2A, lane 4; Fig. 2B, lanes 3, 8, and 13). The addition of primer-template DNA (Fig. 2, A, lane 5, and B, lanes 4, 9, and 14) seems to reverse the...
observed conformational changes as well as the stronger binding interaction between the clamp and the clamp loader (as demonstrated by more cross-linking), again in agreement with our previous fluorescence results (21).

The Arrangement of Subunits within the gp45-gp44/62 Complex in the Absence and Presence of Various Nucleotide Cofactors—These results can provide a rough picture of the arrangement of subunits in the gp45-gp44/62 multiprotein complex. Initially, the gp44/62 binds to the gp45 trimer in a way that brings both the gp44 and gp62 subunits close to the hole in the center of the gp45 ring. There is an asymmetry to this complex, since the three Cys-19 residues of the trimer appear to be nonequivalent in their ability to contact either gp44 or gp62. Instead, only one of the labeled Cys-19 residues seems to be able to contact the gp62 subunit (and possibly gp44), while the other two Cys-19 residues contact only gp44 subunits. Each of the three gp45 subunits appears to be able to contact gp44 equally well at different points, or (perhaps more likely) each gp45 subunit can contact gp44 subunits at specific and different points. gp62 is still near enough to the center of the ring to contact one Cys-19 residue in the presence of ATP, but the gp44 subunits appear to have moved relative to the gp45 trimer so that only one of the previous contact points is maintained, while the other such contact may have been replaced by a different gp45-gp44 contact, as shown by the concomitant disappearance of one gp45-gp44 cross-linked product and the appearance of a new one. Since ATP hydrolysis takes place during the cross-linking reaction, the cross-linking pattern obtained probably does not represent a single protein species. Nonetheless, these data are clear evidence for a conformational change in the presence of ATP, as suggested by our previous fluorescence results (21).

To gain a more complete topographical picture of the conformational changes occurring during ATP hydrolysis by the gp44/62-gp45 system, we have performed identical cross-linking experiments with the gp45K81C mutant, in which the labeled Cys-81 residue is located near the subunit interface on the outside of the ring. Cross-linking of the TFPAM-3-modified gp45K81C mutant leads to the formation of cross-linked gp45 dimer and trimer bands (Fig. 3A, lane 2). A gp45 trimer band could result from the cross-linking of two, or perhaps of all three, labeled residues to nearby gp45 subunits. In the former case, this would lead to the formation of a linear trimer; in the latter, a ring-closed trimer would result. These two differently cross-linked trimer species would be expected to migrate differently on the gel, with the ring-closed trimer migrating more rapidly than the more extended linear form.

A ring-closed trimer is also expected to be the least probable cross-linked species to be formed, since all three labeled residues must simultaneously be close enough to cross-link to their respective neighboring subunit upon photocativation. Since a fourth band indicative of ring-closed trimer formation is not seen, we cannot rule out the possibility that the two types of cross-linked trimers run very close to one another. However, it is more likely that the efficiency of ring-closed trimer formation is very low, perhaps because cross-linking three gp45 subunits through two of the labeled residues prevents the third label from reaching a nearby gp45 subunit. Sexton et al. (22) have previously proposed the existence of an equilibrium between closed and open gp45 ring conformations. Such an equilibrium model could also explain the absence of the ring-closed cross-linked trimer, if we assume (as above) that the cross-linking of two of the three interfaces favors an open conformation at the non-cross-linked interface.

In the absence of nucleotide, Cys-81 contacts only the gp44 subunit (Fig. 3B, lanes 3, 6, and 10). The apparent molecular weights corresponding to the more intense cross-linked bands (bands c, d, and e) that run above the gp45 trimer band are consistent with two or three gp45 subunits cross-linked to one gp44 subunit. Two very faint cross-linked products (bands a and b), which run below the cross-linked trimer, might be the result of one gp45 subunit cross-linked to one gp44 subunit. The cross-linking efficiency for forming these bands appears to be much less when compared with the other bands observed. This suggests that at least two gp45 subunits must be cross-linked to one another before a second labeled residue can reach the gp44 subunit.

A decrease in both the number and the intensities of the cross-linked gp45-gp44 products is observed upon the addition of ATP (Fig. 3A, lane 4, and B, lanes 4 and 7). Since the earlier fluorescence studies have shown the formation of a very tight gp45-gp44/62 complex in the presence of ATP, we believe that the decreased cross-linking observed here reflects a conformational change that places Cys-81 beyond the reach of some contacts that could previously be made and slightly weakens the remaining contacts of Cys-81 with the gp44 subunits. In addition, Western blot analysis reveals a new contact between Cys-81 and the gp62 subunit (Fig. 3B, lanes 4 and 11). This conformational change is partially dissipated upon the addition of primer-template DNA, in that the gp45-gp62 products disappear and some of the gp45-gp44 cross-linked products (bands d and e) are more similar in intensity to those obtained when only the the protein components of the complex were present (without ATP or DNA).

In the presence of ATP (or ATP and DNA), assuming that ATP hydrolysis leads to an increase in the putative opening of the gp45 ring, one would expect to see a decrease in the intensity of the trimer and dimer bands on purely statistical grounds. However, our data cannot unambiguously demonstrate such a change. Since the contacts of the labeled gp45 residues with gp44 subunits become weaker in the presence of ATP, one possibility is that cross-linking to gp44, which competes with gp45 intersubunit cross-linking, is reduced. This might result in a higher efficiency of intersubunit cross-linking and, in turn, might mask ring opening. Similarly, if only one interface is opened, this might also increase the cross-linking efficiency of the other two interfaces. Finally, a third possibility is that the opening process is very fast and is therefore kinetically unobservable. For example, one could assume that the opening is caused by the movement of the gp62 subunit toward an interface, so that gp62 now contacts Cys-81. The gp45-gp62 cross-linked products are very faint in the silver-stained gel and become visible on the Western blot only after long exposure. This low cross-linking efficiency might derive from a weak contact between the two subunits, but it could also derive from a very fast process. If the latter is the case, such a small change in trimer band intensity would not be seen.

The results with the gp45K81C mutant extend this idea of the conformational changes seen with ATP hydrolysis. Initially, the outside of the gp45 ring close to the subunit interface does not contact the gp62 subunit of the gp44/62 complex, and in view of the above observation that the gp62 subunit contacts Cys-19, the gp62 subunit must be near the central hole (not the edge) of the gp45 ring in the absence of nucleotide. Additionally, this labeled gp45 mutant forms cross-linked dimers and trimers upon irradiation. This is expected, since Cys-81 is close to the gp45 subunit interface in the trimer. Upon the addition of ATP, a striking change is observed in the cross-linking pattern; the gp62 subunit now comes into contact with the outside of the gp45 ring, indicating a gross movement of either the gp62 subunit or the gp45 trimer. Other changes are observed in the contacts of the
result of a mismatch between the 3-fold symmetry of the gp45 and gp44/62 (29). This asymmetry in the placement of the trimer to the gp46 subunit, have been observed. Fluorescence experiments using the same mutant (21) failed to reveal any conformational change. However, fluorescence anisotropy studies did indicate some gp44/62-gp45 complex formation in the presence of ATPγS. One possible explanation for this discrepancy is that, at the concentration used in the fluorescence studies (100–200 nM, compared with 1 μM used in the present experiments), the number of gp45-gp44/62 complexes formed was too small for the conformational change to be seen. If this is true, then ATP hydrolysis must be required to stabilize the conformational change, leading to more complex formation. Since ATP and ATPγS bind with similar affinities to gp44/62 (17), the stabilization of the conformational change must occur after binding of the nucleotide substrate.

Very similar cross-linking patterns are seen in the presence of ATP and ATP plus EDTA, in terms of the intensity of the gp45-gp44 cross-linked product bands, when the K81C mutant is used (Fig. 5A, lanes 4 and 6). However, here the cross-linked gp45-gp62 bands appear only in the presence of ATP (Fig. 5B) and not in the presence of ATP plus EDTA or the other nucleotides tested. This suggests that although a similar conformational change is induced when the ATP is bound (and not hydrolyzed), ATP hydrolysis is subsequently required to cause Cys-81 to contact the gp62 subunit. We also note that ATPγS leads to a slightly different cross-linking pattern when compared with the ATP plus EDTA sample (the cross-linked gp44-gp45 products are less intense and the gp45 trimer is more intense in the ATPγS experiment), suggesting that ATPγS and ATP in the presence of EDTA may bind in somewhat different ways.

The effect of ADP was investigated to elucidate further the topography of the gp45-gp44/62 complex when gp44/62 is in the product-bound state. The results from both mutants show that a unique conformation is assumed by the gp45-gp44/62 complex in the presence of ADP that is manifested by a number of cross-linked products and relative band intensities differing from those seen in the absence or the presence of other nucleotides (Fig. 4, lane 6; Fig. 5A, lane 7). In addition we note that the overall cross-linking intensity, especially with the K81C mutant, does not decrease when compared with that seen with only the two protein complexes present (without any nucleotide). Although the interpretation of cross-linking data are complicated by possible different cross-linking efficiencies in different conformational states, these data do not suggest that the ADP-bound state might play a role in dissociating the two protein complexes at the protein concentrations used in this experiment (which are close to those estimated for the physiological situation).

Equilibrium fluorescence experiments provide complementary evidence for the conclusion that the conformation of the gp45-gp44/62 complex depends both on the nature of the nu-
cleotide cofactor and on how it is added. In particular, no change in the intensity or position of the fluorescence peak is observed (data not shown) when ADP is added directly to a mixture of fluorescent site-specifically labeled gp45S19C and gp44/62. However, when an excess of ATP is added to this solution, the fluorescence peak increases to the level characteristic of the ATP-induced state (see Ref. 21). Furthermore, fluorescence polarization experiments indicate that labeled gp45S19C and gp44/62-ADP form a complex characterized by a $K_d$ of $30 \text{ nM}$ (data not shown). Thus, under the conditions of these fluorescence experiments, the environment of the gp45 fluorophore is unchanged when most of the available gp45 is bound to gp44/62-ADP. In contrast, when the gp45-gp44/62-ADP complex is created through the process of ATP hydrolysis, the environment of the fluorophore is dramatically changed. These experiments demonstrate that the ATP hydrolysis event that generates bound ADP must induce a conformation within the gp45-gp44/62 complex that is distinct from that formed when ADP is added to the unliganded proteins directly.

The similarity of the cross-linking patterns obtained in the presence of ATP and in the presence of ATP plus EDTA was not expected. Since the hydrolysis of ATP leads to an ADP-bound state of the complex, one might expect that the pattern with ATP would resemble the pattern obtained with ADP (to the extent that the ADP-added pattern conforms to that which would apply if the ADP-bound complex were formed by ATP hydrolysis; see above), which is clearly not the case for either mutant. If the cross-linking efficiencies of the different states are comparable, a possible resolution is that, in the presence of ATP, most of the gp45-gp44/62 complexes formed exist in the ATP-bound state. This would mean that ATP hydrolysis is a slow step in the reaction. We note that the cross-linking patterns of the gp45-gp44/62 complexes observed with both mutants in the presence of both ATP and DNA are more similar to the cross-linking patterns in the presence of ADP, suggesting that ATP hydrolysis is faster in the presence of DNA and therefore that the ADP-bound state is more easily detected under these conditions.

A Model of the Overall Clamp Loading Cycle—Our presently preferred interpretation of the cross-linking results discussed above, combined with the results of previous fluorescent and kinetic studies (12, 13, 21, 22), is summarized in the model shown in Fig. 7. The binding of ATP to the gp45-gp44/62 complex is sufficient to induce a conformational change. However, gp62 contacts Cys-81 only in the complex produced by ATP hydrolysis. Therefore, ATP hydrolysis causes a particular change in conformation that puts the Cys-81 residue in contact with gp62. This relative movement of the subunits in the gp44/62-gp45 complex might be related to the opening of the ring. The clamp loading complex in the presence of ATP (and in the absence of DNA), as visualized in studies with both mutants, is very different from that seen in the presence of ATP and DNA, in agreement with ADP release being at least partially rate-limiting. Once ADP is released from the complex, the cycle can start again.

**Fig. 7. Proposed mechanism for the loading of the gp45 sliding clamp onto primer-template DNA.** At physiological concentrations of ATP and proteins, a very stable gp45-gp44/62 complex is formed, in accord with results presented here combined with previous fluorescence data (21, 22). The gp45-gp44/62 complex hydrolyzes ATP in a relatively slow manner, as shown by the similar cross-linking patterns obtained in the presence of ATP and ATP plus EDTA. In the presence of primer-template DNA, ATP hydrolysis of the gp45-gp44/62 complex is stimulated severalfold (7, 10, 12, 13). We propose that the ATP hydrolysis step is accelerated when the gp45-gp44/62-ATP complex encounters a primer-template junction and, as a consequence, the ring is rapidly opened. The proximity of DNA might favor the reclosing of the ring around the DNA by electrostatic interactions. This should also favor the dissociation of the gp45 ring from the gp44/62 complex. Once loaded onto the DNA, the gp45 ring would rapidly slide off the DNA and be free to rebind to the gp44/62 complex, even if ADP is still bound to it, according to our cross-linking experiments that show that a unique complex is formed in the presence of ADP. In addition, the similarity of the cross-linking patterns suggests that the same complex is mostly seen in the presence of ATP and DNA, in agreement with ADP release being at least partially rate-limiting. Once ADP is released from the complex, the cycle can start again.
teins form a very stable complex that hydrolyzes ATP relatively slowly at physiological concentrations of ATP and proteins and that the ATP hydrolysis step is accelerated when the complex encounters a primer-template DNA junction. The fast hydrolysis step would immediately lead to the putative ring-opening step. Upon hydrolysis, the proximity of the DNA might induce the ring to reclose around it, perhaps driven by the electrostatic interaction between the negative charges on the DNA and the positive charges inside the gp45 ring. This might, in turn, favor the dissociation of the gp44/62 complex from the ADP-bound gp44/62 complex. The overall cycle can then begin again once ADP is released from the gp44/62 complex. Additional structural features of the clamp loading cycle are illuminated by the experiments presented in the companion papers (29, 31).

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