Photo-induced cross-linkage of gene-5 protein and bacteriophage fd DNA

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ABSTRACT

The gene 5 protein, coded for by the bacteriophage fd, forms a complex with single stranded fd-DNA such that one gene 5 protein monomer interacts with four bases. Exposure of this complex to ultraviolet light results in the formation of covalent bonds between 25-30% of the gene 5 protein monomers which are bound to the DNA. In contrast, when the intact fd virion, which is a complex of coat protein and DNA, was exposed to ultraviolet irradiation, no detectable protein DNA cross-links were found.

INTRODUCTION

The male specific, filamentous coliphage fd infects its host non-lethally, producing 200-2000 infectious particles per bacterial generation [1]. At an intermediate stage of viral morphogenesis, the virus encoded product of gene 5 (gene 5 protein) mediates conversion of DNA replication from synthesis of double-stranded replicating form II to synthesis of viral single strands, [2,3,4,5] forming an intracellular complex with viral strands prior to the displacement of gene 5 protein by coat protein to form the intact virion.

The gene 5 protein fd-DNA complex has a number of advantages for the study of protein-DNA interactions. The primary structure of gene 5 protein is known [7], the conformational states of the fd DNA are being studied [8] and the photochemistry of the gene 5 protein-fd DNA complex is currently under investigation. The information provided by this work should permit a precise stereochemical interpretation of the results that can be obtained with this system. Physical and chemical methods are being used to determine the involvement of certain amino acid residues of the gene 5 protein in its binding to DNA [8]. X-ray diffraction studies of the fd bacteriophage have also clarified the arrangement of the coat protein subunits in the virus but they have not as yet provided
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enough information to enable one to discriminate among several models which have been proposed for the secondary structure of the viral DNA or for the molecular details of its interaction with either the coat or the gene 5 protein [9]. Characterization of cross-linked coat protein or gene 5 protein-viral DNA complexes, if they could be obtained, would yield important information about the relative orientation of photoreactive DNA bases to particular amino acid residues in the surrounding protein subunits.

In this paper we report our attempts to introduce covalent linkages between gene 5 protein, coat protein, A protein and fd viral-DNA by ultra-violet irradiation of the gene 5 protein-fd DNA complex and of the intact virion. The relative efficiencies of photo-induced cross-linking in these two systems were determined. The gene 5 protein-fd DNA complex was rapidly cross-linked by ultra violet light. About 25% of the gene 5 protein in the complex was covalently linked to the DNA, whereas very little, if any, of the major coat protein or the A protein was covalently joined to the fd viral DNA. These results are briefly discussed in terms of models for the gene 5 protein-DNA complex and for the intact virion.

MATERIALS AND METHODS

Buffers and media. Media N-12 and N-13 used for growing E. coli DM48 have been described previously [10]. Growth medium for large scale preparations contained 30gm Bacto-tryptone (Difco), 20gm yeast extract (Difco) and 5gm NaCl per liter. Standard buffer I contained 10mM Tris-HCl, pH 7.2, 1mM EDTA, 0.1% (V/V) β-mercaptoethanol. Standard buffer II was identical to standard buffer I except that it contained 0.05M NaCl.

Growth and isolation of unlabelled gene 5 protein.

E. coli DM48 [11] cells were grown to a density of about 10^8 cells/ml in tryptone broth and infected with wild type fd bacteriophage [12] at a multiplicity of 10 to 1. Six hours after infection, cells were harvested and resuspended in 1-2 volumes of standard buffer I. Cells were lysed by grinding with an equal mass of fine glass beads in a Waring blender, and incubated with about 10 μg/ml pancreatic DNAase (Worthington) in solutions containing 20mM MgCl_2 and 5mM CaCl_2 for one hour at 4°. The mixture was precipitated by adding NaCl and ammonium sulfate to give final concentrations of 0.5M and 25% respectively, incubated one hour at 4°, and centrifuged at 10,000 rpm in a Sorvall SS-1 rotor for 60 minutes. The pellet was resuspended in standard
buffer I. Treatment with DNAase, salt precipitation, pelleting and resuspension were repeated as described above. The resuspended protein solution was made 10mM in EDTA, poured over a single-stranded, salmon sperm DNA cellulose column prepared as described by Alberts and Herrick [13], washed with several column volumes of standard buffer II followed by standard buffer I containing 0.15M NaCl, and finally eluted with standard buffer I containing 0.6M NaCl. The protein was concentrated by adding ammonium sulfate to a final concentration of 25%, incubating at 4°C for one hour, and centrifuging as described above. The pellet was resuspended in standard buffer I at 3-8mg/ml and frozen at -70°F.

Preparation of radioactive labelled Gene 5 Protein.

For the preparation of radioactive gene 5 protein, cells were grown at 37°C in N-13 medium containing 0.01% casein amino acids. When the bacteria reached a density of 10^8 cells/ml they were infected at a multiplicity of 100 to 1 with fd phage. A mixture of ^3H-amino acids having specific activities of 20-50 μCi/ml or ^14C-amino acids at (2-5 μCi/ml) was added immediately after infection and the culture was maintained under these conditions for 90 minutes. Cells were then harvested by centrifugation. Lysozyme at a concentration of 330 μg/ml in standard buffer I was added to disrupt the cells, and isolation of labelled gene 5 protein was performed as described above, except that only one treatment with DNAse was used and only one ammonium sulfate precipitation was employed. 100 μg carrier gene 5 protein was added to the mixture after DNAse treatment and before precipitation with ammonium sulfate.

Preparation of fd phage and fd viral-DNA

Purified fd phage and fd viral DNA were prepared following the method of Marvin and Schaller [14], except that phage were initially precipitated from harvested growth medium by the addition of NaCl to give a concentration of 0.05 M and polyethylene glycol to a final concentration of 3%. After standing for 2 hours at 4°C, the phage was centrifuged for 20 minutes at 6,000 rpm in an SS-1 Sorvall rotor.

^32P-labelled fd phage and fd viral DNA were prepared using the method of Tseng and Marvin [15].

fd phage and fd viral DNA, labelled with ^3H-thymidine, were isolated from cultures of E. coli grown in N-12 medium and infected as described for the preparation of radioactive gene 5 protein. Immediately after infection, 20 μCi/ml of ^3H-thymidine was added, and the culture allowed
to grow for another 6 hours. Isolation of labelled phage and DNA were

carried out as described for the unlabelled preparations.

fd phage labelled with $^{14}$C-amino acids was prepared from 5 ml
cultures which were grown, infected, and labelled as described for
radioactive gene 5 protein preparations. The phage was precipitated
with NaCl and polyethylene glycol as before, resuspended in 0.1 ml of
standard buffer II, and layered on a 4.5 ml, 5-20% linear sucrose gradient
in standard buffer II. After sedimenting 90 minutes at 40,000 rpm
(Spinco SW50.1 rotor), the rapidly sedimenting (40S) material was
collected, dialyzed against 200 volumes of standard buffer II, and
stored at 4°C.

Photolysis and phenol extraction

Samples for irradiation (0.1 ml) were placed 6 cm from the center
of a 15W (GE) germicidal lamp, main output 253 nm dosage: 476 erg/mm$^2$/sec,
and exposed at room temperature for various times. The samples were
then diluted to 1 ml with 0.08 M Na$_2$B$_4$O$_7$ (pH 8.1) and extracted by
shaking with 1 ml redistilled phenol saturated with the 0.08 M borate
buffer. After centrifugation at room temperature (1,500 rpm, Sorvall
SS-1 rotor, 10 minutes) the aqueous layer was removed, and was freed of
material which had precipitated at the interface. Half of the aqueous
phase was counted and the remainder reextracted with 0.5 ml of fresh
phenol as described above. The entire aqueous layer was removed and
counted. $^{32}$P labelled fd DNA, gene 5 protein, and bovine serum albumin
were used in the buffers indicated and in concentrations given in table 1.

Preparation and gel electrophoresis of DNAse-generated fragments
from the gene 5 protein-DNA complex and the fd virion before and after
photolysis.

One tenth of a milliliter of solutions containing 110 ng/ml $^3$H-
-thymidine labelled fd DNA and 3.6 μg/ml of gene 5 protein were mixed,
one portion exposed to ultraviolet light for 1 hour as described above
and the other portion kept in the dark. This procedure was followed by
addition of 0.1 ml of a solution containing 28 μg/ml of unlabelled fd
DNA. The mixture was incubated for one hour at 37°C to allow exchange of
most of the protein from labelled to unlabelled DNA. At a gene 5
protein-fd DNA mass ratio of about 7/1 at saturation [16,17], the cold
DNA was in 30-fold in excess over that needed to complex the amount of
gene 5 protein present. MgCl$_2$ and CaCl$_2$ were added to give a final
centreration of 20 mM and 5 mM respectively, and the mixture was incubated
Table 1. DNA recovery after phenol extraction

<table>
<thead>
<tr>
<th>Sample (concentrations in mg/ml)</th>
<th>Molar Concentration of NaCl in standard buffer</th>
<th>Irradiation (concentrations of NaCl in standard time (min.) or erg/mm²)</th>
<th>Percent DNA recovered in the aqueous phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA gene 5 protein (Non-dissociating conditions)</td>
<td>0.05</td>
<td>0.2</td>
<td>0.05</td>
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<tr>
<td></td>
<td>0.05</td>
<td>0.2</td>
<td>0.05</td>
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<tr>
<td></td>
<td>0.2</td>
<td>0.7</td>
<td>0.05</td>
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<tr>
<td></td>
<td>0.2</td>
<td>0.7</td>
<td>0.05</td>
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<td>2.0</td>
<td>7.0</td>
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<tr>
<td></td>
<td>2.0</td>
<td>7.0</td>
<td>0.05</td>
</tr>
<tr>
<td>DNA gene 5 protein (Dissociating conditions)</td>
<td>0.05</td>
<td>0.2</td>
<td>1.0</td>
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<td></td>
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<td>0.2</td>
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<tr>
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</tr>
<tr>
<td>fd DNA BSA</td>
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<td>70</td>
<td>0.05</td>
</tr>
</tbody>
</table>

1. All samples contained 70 µg/ml of bovine serum albumin (BSA) except when fd DNA was irradiated alone.

for 30 minutes at 37° with 10 µg/ml of pancreatic DNAse. After DNAse treatment the sample was made 1% in SDS and run on a 10 cm 10% polyacrylamide gel in the presence of SDS [18], for 2.5 hours at 10 mA/tube. The gel was sliced into 3mm sections, crushed [19] and counted using 3 ml Triton X-100 scintillation fluid per sample (Fig 1).

RESULTS

In an attempt to find out whether gene 5 protein, fd coat protein or A protein could be cross-linked to fd viral-DNA, we used a simple assay which involved counting 32P labelled fd DNA in the aqueous phase after phenol extraction of the irradiated gene 5 protein-32P-fd-DNA complex or the 32P-labelled fd virion. The radioactivity remaining in the phenol-extracted aqueous phase, after irradiation, was compared with the radioactivity found in the aqueous phase of the unirradiated control. The results shown in table I can be summarized as follows: Irradiation of the gene 5 protein-fd DNA complex for a short time causes the fd-DNA to lose its solubility completely in the aqueous phase. The loss of solubility of the DNA is independent of the absolute concentrations of DNA and gene 5 protein (within the ranges that were tested) providing
that stoichiometric amounts of gene 5 protein (one monomer per four nucleotides) are used. When gene 5 protein and fd DNA were irradiated under dissociating conditions (1M NaCl), the amount of DNA remaining in the aqueous phase was dependent on the absolute concentrations of the protein and the DNA. At the high end of the concentration range most of the DNA became insoluble. Irradiation of the fd virion did not result in appreciable loss of DNA solubility. The other controls, exposure to ultraviolet light of fd-DNA alone and in the presence of bovine serum albumin, under different conditions, did not produce a significant decrease in the solubility of the DNA. The variability in the observed recoveries of $^{32}$P counts can be attributed to a number of different factors. The largest errors probably result from the small volumes used and the fact that phenol-water emulsions were not broken completely in all cases.

Although the results of phenol extraction suggested that cross-linking between gene 5 protein and fd DNA occurred, it was necessary to get more direct evidence. This was done in two ways: 1) by demonstrating that DNAase treated fragments of $^{32}$P labelled fd-DNA from the irradiated gene 5 protein-fd DNA complex had a mobility in SDS-polyacrylamide gel electrophoresis consistent with the molecular weight of the gene 5 protein (10,000 daltons) and; 2) by showing that labelled gene 5 protein and fd-DNA, after irradiation, cosedimented in a sucrose gradient under dissociating conditions.

The gene 5 protein-fd DNA complex (labelled with $^3$H-thymidine) was first irradiated, then incubated with excess unlabelled fd DNA to competitively remove non-covalently bound gene 5 protein from the irradiated, gene 5 protein-$^3$H-fd DNA complex. This mixture was then treated with DNAase in the presence of $^{32}$P-fd DNA (as a marker) and run on SDS acrylamide gel electrophoresis giving the patterns shown in Fig 1. In the irradiated sample, (solid line) half of the DNA was found in the higher molecular weight region and the other half appeared at the gel front. In contrast, nearly all of the DNA from the non-irradiated gene 5 protein-fd DNA complex (dashed line) was found at the front of the gel. When fd DNA, alone was irradiated, (semi-dashed curve) 90% of the input counts appeared at the gel front and 10% of the input counts spread throughout the gel. We interpret the results, shown in Fig. 1, as indicating that irradiation produces covalent bonds between fd DNA and gene 5 protein since the presumed cross-linked gene 5 protein-$^3$H-fd DNA fragments, produced by DNAase treatment, would be expected to migrate close to the
position found for gene 5 protein in the gels (results not shown).

The presumed photo-induced covalent linking of gene 5 protein to fd DNA suggested by the results of the experiment just described, was confirmed by finding that $^{14}$C-labelled protein, and fd DNA from the irradiated complex, cosedimented in a sucrose gradient under dissociating conditions. Thus, when gene 5 protein and fd DNA were mixed at a 7 to 1 mass ratio of protein to DNA, irradiated for 15 minutes and sedimented through a sucrose gradient containing 0.6M NaCl, 37% of the protein cosedimented with the DNA (Fig.2c). Size heterogeneity of the irradiated complex (as evidenced by material trailing in the gradient) was also observed. When a shorter irradiation time (5 minutes) was used 30% of the protein sedimented with the DNA, and less fragmentation of the
complex occurred (results not shown). In a control experiment, the amount of DNA and protein cosedimenting in the absence of irradiation was found to be 11% (Fig. 2a). Irradiation of fd DNA alone for 15 minutes resulted in extensive fragmentation (Fig. 2b).

The assumption that cosedimentation of the gene 5 protein and the fd DNA after irradiation is due to formation of stable covalent linkages and not to gene 5 protein aggregates, that just happen to cosediment with the DNA, is supported by the observed shift in the original position of the fd DNA control (Fig. 2a) to a higher S value after photolysis of the protein-DNA complex (Fig. 2c). The same shift in the sedimentation coefficient of fd DNA is observed when gene 5 protein forms a complex with fd DNA and is sedimented under nondissociating conditions [6,16,17].

In view of the relatively high efficiency of cross-linking between the gene 5 protein and fd DNA, (the minimum quantum yield based on the assumption of 1 hit per protein monomer was about 2 x 10^-4 ) it was of interest to see if the fd coat protein could also be cross-linked with comparable efficiency to fd viral DNA. Experiments employing the phenol extraction assay suggested that some cross-linking might have occurred after ultraviolet irradiation of the virion for 1 hr. In an attempt to estimate the amount of fd coat protein linked to DNA, fd phage labelled with 32p-DNA and 14C-coat protein was exposed to ultraviolet light and sedimented through a sucrose gradient under dissociating conditions. Without irradiation 4.1% of the 14C-coat protein label sedimented with viral DNA (Fig. 3a). When the virion was irradiated for increasing lengths of time (Fig. 3b and 3c), there was no significant increase in the proportion of 14C label cosedimenting with viral DNA, but the sedimentation pattern of the DNA indicated that fragmentation of the virus particle had occurred. Since we could have detected a 2% difference in the amount of coat protein cosedimenting with viral DNA, it is possible that a maximum of 60 coat protein subunits could have been covalently attached to the fd DNA, and if this had occurred, it might have been sufficient to reduce the amount of fd DNA recovered in the aqueous phase. However, the length of photolysis time (1 hr.) required before significant loss of DNA is observed, makes it likely that the low recovery is due to non-specific photochemical reactions between the protein and DNA.

In addition to the major coat protein, the fd virion contains 3 or 4 copies of a high molecular weight protein (the A protein, a product of
gene 3 [20]) and it was of interest to see whether ultraviolet irradiation of the fd virion could produce stable A protein-DNA cross-linkages. This was tested using fd virus labelled with $^3$H-histidine (an amino acid found in the A protein but not in the coat protein). Since no significant difference was found in the amount of tritium sedimenting with viral DNA before and after 15 minutes of photolysis we concluded that no A protein-

![FIG. 3](image)

**FIG. 3** Sucrose density gradient sedimentation of the fd virion before and after irradiation.

The virus, at a concentration of 0.1 mg/ml, was irradiated as described in MATERIAL AND METHODS and placed in a dissociating buffer consisting of 1 M urea, 20 mM sodium acetate, 20 mM EDTA and 40 ml 91F HCl pH 7.2. After standing for 30 minutes, the samples were layered on a 4.5 ml, 5-20% linear sucrose gradient which also contained the components in the dissociating buffer at concentrations one half of those listed above. The samples were centrifuged for 4 hours, 40,000 rpm in a Spinco SW50.1 rotor at 18°C. Fractions of 0.2 ml were collected from the bottom of the tube (at the left of the figure) and counted in 3 ml of Triton X-100 acetone solution. All counts were corrected for channel overlap. The solid line (---) represents $^3$H-fd DNA. The dashed line (0-0-0) represents $^3$C coat protein. Panels a, b and c show the distribution of counts at times 0, 5 and 60 minutes after irradiation of the virus.

fd DNA cross-links were formed.

**DISCUSSION**

It was observed some time ago that the recovery of DNA, after phenol extraction of DNA-protein complexes is markedly reduced after irradiation with ultraviolet light [21]. A number of amino acids form adducts with uracil after exposure to ultraviolet light [22,23]. Subsequently, the technique of ultraviolet induced cross-linking has been used to generate covalent bonds between E. coli lac repressor and BrdU-Δh80 DNA [24], E. coli DNA and DNA polymerase [25] and between poly d (A-T)-d(A-T) and RNA polymerase [26].

The results of the experiments reported here provide evidence for the formation of covalent bonds between fd DNA and roughly one quarter of the saturating level of gene 5 protein, (37% minus 11% for the non-irradiated control) after short exposure to ultraviolet radiation. This corresponds to about 400 gene 5 monomers for each fd viral DNA strand of 6000 bases. In contrast, virtually no cross-linking occurred between
the coat or the A protein and fd viral DNA after irradiation. The absence of any concentration dependence on the efficiency of cross-linking of gene 5 protein to fd viral DNA supports the idea that there is tight binding as well as suitably juxtaposed amino acid residues and bases which permit the formation of photo induced DNA-protein crosslinks, even without BrdU present in the DNA. Neither the fd coat protein, the A protein nor bovine serum albumin will form covalent linkages with fd viral DNA upon irradiation. When the gene 5 protein and fd DNA are photolyzed under conditions which favor dissociation of the protein DNA-complex, the amount of DNA cross-linked is dependent on the absolute concentrations of protein and DNA indicating that some association occurs even in relatively high salt concentration. The fact that crosslinkage is not complete at high DNA-protein concentrations is probably due to competing photo-inactivation processes which occur more rapidly relative to crosslinking. Although the interaction of gene 5 protein with DNA is not dependent on the DNA sequence there may be certain bases which can be more readily cross-linked than others. We are testing this notion using radioactive gene 5 protein and the four homopolymers.

The striking difference between the behavior of gene 5 vs. coat protein, in their complexes with DNA, may reflect the relative disposition of photochemically reactive amino acid side chains or DNA bases with respect to one another in the gene 5 protein fd-DNA complex compared to their proximity in the intact virion. If this is true, one would expect significant differences in the molecular architecture of the two nucleoprotein complexes. Circular dichroism and spectral studies have shown that three of the five tyrosine residues in the gene 5 protein shift from a more hydrophilic to a more hydrophobic environment when the protein forms a complex with fd DNA [27]. Recent studies with chemically modified gene 5 protein have provided evidence for intercalation of three tyrosyl side chains into the fd single stranded DNA [8]. These results suggest that tyrosine residues may also be involved in the photo-induced cross-linking of gene 5 protein to fd-DNA, a view consistent with the known photochemical reactivity of tyrosine residues toward nucleic acids [22] and with our observation that cross-linking in the gene 5 protein-fd DNA complex proceeds with a high efficiency. No evidence exists for intercalation of amino acid residues of the coat protein and fd DNA in this virion. Studies are currently in progress to identify the amino acid residues in the gene 5 protein and the bases in fd DNA that are covalently joined. It is hoped that these results will lead to an understanding of the
general requirements that have to be met for the photochemically induced linking of this class of proteins and DNA to occur.

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